Microbial nitrogen cycle interactions in laboratory-scale model systems

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Content

Anaerobic oxidation of ammonium (anammox) is a microbial process playing an important role in the recycling of fixed nitrogen on a global scale, converting ammonium and nitrite into dinitrogen gas. In natural aquatic ecosystems anammox bacteria are generally limited in one or both of their substrates as ammonium and nitrite are (intermediate) products that are constantly and rapidly turned over in several biogeochemical processes. Therefore they are often dependent on other nitrogen-transforming processes for the supply of their substrates. The interaction of anammox bacteria with their environment and the different aspects that might play a role therein are the topic of this thesis. A special focus will lie on the acquisition of nutrients derived from other N-transforming processes and further research on the versatile metabolism of anammox bacteria from which they might benefit under nutrient scarcity they encounter in the environment.

In **Chapter 2** we studied the diversity and abundance of anammox bacteria in cold seep sediments and hydrothermal vent areas at the Guaymas Basin in the Cortés Sea using unique anammox biomarkers, i.e. the alpha subunit of the hydrazine synthase (*hzsA*) and core ladderane lipids. These systems might be suitable habitats for the anammox bacteria but this has not been investigated in detail. All *hzsA* clones retrieved were closely associated to the '*Candidatus* Scalindua' genus. The analysis of this phylomarker revealed a very high diversity within the genus Scalindua. Comparison of individual sequences showed that several of these had a similarity as low as 76% on nucleotide level. Absolute numbers of anammox bacteria in the sediments samples were determined by amplification of a 257 bp fragment of the *hszA* gene in a qPCR assay showing that numbers of anammox bacteria were up to 90% higher in cold hydrocarbon-rich sediments compared to the vent areas and the reference zone. Ladderane lipids, unique to anammox bacteria, were also detected in several of the sediment samples and these were in line with the *hzsA* analysis. Due to the high concentration of reduced sulfur compounds and its potential impact on the cycling of nitrogen (see also Chapter 3) we aimed to get an indication about the key players in the oxidation of sulfide in the Guaymas Basin sediments using the alpha subunit of the adenosine-5'-phosphosulfate (APS) reductase (*aprA*). Amplification of this gene revealed a high number of gammaproteobacterial *aprA* sequences covering the two sulfur-oxidizing bacteria *aprA* lineages as well as sulfate-reducers.

Chapter 3 focusses on the interaction between anammox bacteria and microorganisms able to oxidize reduced sulfur compounds in more detail. The coupling of sulfide-dependent denitrification to anammox is particularly interesting because besides hydrogen, sulfide is the most important reductant

at the chemocline of anoxic marine basins and is abundant within sediments. We first showed that micromolar concentrations of sulfide inhibited anammox activity. Based on this result, we investigated whether the anammox process could be linked to autotrophic denitrification under continuous sulfide flux, where the *in situ* sulfide concentration in the reactor was kept below the detection limit (5 μM). In such a system, a denitrifier would reduce nitrate to nitrite while oxidizing sulfide and thereby supply anammox bacteria with one of its substrates. To test this hypothesis a laboratory scale model system containing a co-culture of anammox bacteria and the autotrophic denitrifier *Sulfurimonas denitrificans* DSM1251 was started. Complementary techniques revealed that the gammaproteobacterial *Sedimenticola* sp. took over the intended role of *Sulfurimonas denitrificans*. A stable coculture of anammox bacteria and *Sedimenticola* sp. consumed sulfide, nitrate, ammonium and CO_2. Anammox bacteria contributed 65-75% to the nitrogen loss from the reactor. The cooperation between anammox and sulfide-dependent denitrification may play a significant role in environments where sulfur cycling is active and where actual sulfide concentrations stay below μM range. The reactor model system not only allowed the study of the physiology of anammox and an interaction partner, but it also represented a convenient way to study the effect of differential limitations of substrates as they could occur in nature on a gene expression level (**Chapter 4**). To investigate the response of anammox and autotrophic denitrifiers to nitrate- and ammonium-limiting conditions we subjected the co-culture to substrate limitation and compared the change in the transcriptome of the involved microorganisms (RNAseq) to a standard condition. Under ammonium-limiting conditions 762 genes of the *Scalindua* sp. present in the co-culture were either up- or downregulated representing slightly more than 15% of the total predicted genes. As ammonium is an important substrate its low concentrations lead to the downregulation of genes involved in energy conservation, transcription, translation, protein biosynthesis, cell division and carbon fixation. Furthermore, there was a significant increase in the expression of ammonium transporting genes (*amtB*). The most significant upregulation was observed for scal00591 (up to 130x) and scal00596 (up to 16x), which are encoded in a gene cluster together with two other *amtB* genes and two P-II regulatory proteins. The expression of all genes encoded in this cluster increased by 7-fold at least. The two encoded *focA*-like formate/nitrite transporters were also upregulated, replacing the *nirC*-like scal00416 as the most highly expressed nitrite transporter under control conditions in this experiment. Also genes involved in the formation of flagella were highly upregulated under ammonium limitation. Nitrate limitation did not

have the same effect on *Scalindua* sp. as differential expression of only 228 genes (4.6%) was observed. *Sedimenticola* sp. differentially expressed 742 genes under nitrate limitation, which represented 17% of all its genes. The most significant changes in *Sedimenticola* sp. were the downregulation of genes making up the membrane-bound nitrate reductase complex, genes involved in sulfur metabolism as well as energy conservation and central metabolism. Ammonium limitation only resulted in the up- or downregulation of 331 genes (7.5%) including the upregulation of ammonium transporter genes and genes encoding nitrogen assimilation enzymes. This study might help to shed more light on the possible role of unknown enzymes or the physiological role of known enzymes in anammox bacteria and the denitrifying partner by studying first-line gene expression changes in response to ammonium and nitrate limitation.

Whether anammox bacteria might be able to overcome ammonium-limiting conditions by switching to an alternative metabolism was investigated in **Chapter 5**. Amino acids constitute an important fraction of dissolved organic nitrogen in the marine ecosystems and have the potential to serve as additional electron donors in ammonium-liberating processes (denitrification, dissimilatory nitrate reduction to ammonium (DNRA)) as well as act as a direct source of ammonium via deamination. To test the effect of amino acids, *K. stuttgartiensis* was cultivated in a membrane reactor under ammonium limitation and either pulse-fed daily with a mixture of amino acids (glutamate, glycine and serine) or fed continuously with glutamate for 110 days. We monitored nutrient concentrations and used metagenomics to examine the changes in the microbial community. The addition of amino acids stimulated nitrate and nitrite reduction rates and led to ammonium accumulation in the reactor. As a result nitrite became limiting, inducing a competition between anammox and other microorganisms for that substrate. Experiments with ^{15}N-labelled glutamate and glycine showed an increase in $^{29}N_2$, indicating that amino acids could serve as a source of NH_4^+ in the anammox process. Based on these results it could not be concluded however which member of the enrichment culture metabolized amino acids. Metagenomic analysis revealed that amino acid addition supported growth of a denitrifying and/or fermenting side population that accounted for about 40-50% of the reads and was dominated by *Proteobacteria* and *Bacteriodetes*. The stable cooperation between heterotrophic denitrification and anammox could be important in dynamic nitrate-rich systems where organic substrates are not always available in excess.

To study the versatile organic acid oxidation and carbon fixation metabolism

of anammox bacteria in more detail, **Chapter 6** focused the on the metabolism of short chain organic acids. The AMP-forming acetyl-CoA synthetase gene (*acs*) of *K. stuttgartiensis*, encoding an important enzyme involved in the conversion of organic acids, was identified and heterologously expressed in *Escherichia coli* to investigate the activation of several substrates such as acetate, propionate and butyrate. This protein, which is the most highly expressed acetate-activating enzyme (AMP-forming ACS; kustc1128) in *K. stuttgartiensis*, could be involved in the acetyl-CoA pathway, but could also lead to the direct incorporation of acetate by anammox bacteria. The *acs* gene coded for a protein of 589 amino acids, and could complement an *E. coli* triple mutant, deficient in all pathways of acetate activation. The His-tag-purified ACS enzyme showed activity towards acetate, propionate and butyrate and had a K_m of 0.19 mM for acetate. These properties were compatible with mixotrophic growth of anammox bacteria. In addition to *acs*, the genome of *K. stuttgartiensis* contained the essential genes of an acetyl-CoA synthase/CO dehydrogenase complex, and genes encoding two isoenzymes of archaeal-like ADP-forming acetyl-CoA synthetase underlining the importance of acetyl-CoA as intermediate in the carbon metabolism of anammox bacteria.

De anaerobe oxidatie van ammonium (anammox) is een microbieel proces, dat op wereldschaal een belangrijke rol speelt bij het recyclen van in organisch materiaal vastgelegde stikstof. Anammox bacteriën zetten ammonium en nitriet om in stikstofgas. In aquatische ecosystemen zijn ze over het algemeen gelimiteerd in één of in beide substraten, omdat zowel ammonium als nitriet (tussen)producten zijn van andere microbiële processen en vlug omgezet kunnen worden. Dit is de reden waarom anammox bacteriën voor de vorming van hun substraten vaak afhankelijk zijn van andere stikstof-omzettende processen. De interacties tussen anammox bacteriën en hun omgeving en de verschillende aspecten die hierin een rol kunnen spelen zijn daarom onderwerp van dit proefschrift.

In **Hoofdstuk 2** hebben we de diversiteit en kwantiteit van anammox bacteriën in koude submariene en hydrothermale bronnen van het Guaymas Basin in de Cortés Zee bestudeerd door gebruik te maken van unieke biomarkers, zoals de ladderaanlipiden en het gen dat codeert voor de alfa subeenheid van het hydrazine synthase (*hzsA*). Deze ecosystemen zouden geschikt kunnen zijn als habitat voor anammox bacteriën maar dit is nog nooit in detail onderzocht. Alle verkregen *hzsA* klonen behoorden tot het '*Candidatus* Scalindua' geslacht. De analyse van de *hzsA* phylomarker liet een hoge diversiteit binnen het *Scalindua* geslacht zien. Een vergelijking van individuele sequenties toonde aan, dat sommigen een overeenkomst van slechts 76% op nucleotide level hadden. Kwantificering van anammox bacteriën in de sedimentmonsters werd gedaan door middel van amplificatie van een 257 bp fragment van het *hzsA* gen in een qPCR assay. Hierbij bleek dat de absolute aantallen anammox bacteriën tot 90% hoger waren in de koude submariene bronnen ten opzichte van de hydrothermale bronnen en een referentie zone. Ladderaanlipiden, uniek voor anammox bacteriën, werden ook gedetecteerd in een aantal van de sedimentmonsters en de verkregen resultaten kwamen overeen met die van de *hzsA* analyse. Vanwege de hoge concentraties gereduceerde zwavelverbindingen en de potentiele invloed die dit kan hebben op de stikstofcyclus, wilden wij ook een indruk krijgen van de microbiële populatie die sulfide oxideert in de Guaymas Basin sedimenten. Daarom werd een moleculaire analyse uitgevoerd van het gen dat codeert voor de alfa subeenheid van het adenylyl-sulfaat reductase (*aprA*). Amplificatie van dit gen resulteerde in een groot aantal *aprA* sequenties behorende tot twee verschillende *aprA*-afstammingslijnen van sulfide-oxiderende Gammaproteobacteriën. Bovendien werden ook sequenties afkomstig van sulfaat reduceerders gevonden.

Hoofdstuk 3 richt zich op de interactie tussen anammox bacteriën en micro-

organismen die in staat zijn om gereduceerde zwavelverbindingen te oxideren. Het koppelen van sulfide-afhankelijke denitrificatie en anammox is bijzonder interessant, want naast waterstof, is sulfide een belangrijke elektronendonor in zuurstofloze, mariene ecosystemen en is vaak ruimschoots aanwezig in sedimenten. Als eerste lieten we zien dat sulfide concentraties in de orde van een aantal micromolair anammox activiteit remden. Aan de hand van dit resultaat, hebben we onderzocht of het anammox proces alsnog gelinkt zou kunnen worden aan autotrofe denitrificatie door een continue aanvoer van sulfide te koppelen aan een hoge omzettingssnelheid. Daardoor zou het mogelijk moeten zijn om de *in situ* concentraties van sulfide in de reactor onder de detectielimiet (5 μM) te houden. In zo een systeem zou een denitrificeerder nitraat reduceren naar nitriet door sulfide te oxideren en op deze manier anammox bacteriën voeden met één van hun substraten en tegelijkertijd de sulfide concentraties laag houden. Om deze hypothese te testen werd een reactor model systeem opgezet met een co-cultuur van anammox bacteriën en de autotrofe denitrificeerder *Sulfurimonas denitrificans* DSM1251. Met behulp van complementaire technieken konden wij laten zien dat de Gammaproteobacterie *Sedimenticola* sp. al snel de rol van *Sulfurimonas denitrificans* had overgenomen. Uiteindelijk resulteerde dit in een stabiele co-cultuur van anammox bacteriën en *Sedimenticola* sp. die sulfide, nitraat, ammonium en CO_2 consumeerde. Anammox bacteriën waren hierbij verantwoordelijk voor de productie van 65-75% van het vrijkomende stikstofgas. De samenwerking van de anammox bacteriën en de sulfide-afhankelijke denitrificeerders zou een belangrijke rol kunnen spelen in ecosystemen met een actieve zwavelcyclus gekoppeld aan actuele sulfide concentraties lager dan enkele micromolair. Het voor deze studie gebruikte reactormodelsysteem is niet alleen geschikt om de fysiologie van anammox en de denitrificerende partner te bestuderen, het geeft ons ook de mogelijkheid om op een effectieve manier de gevolgen van in de natuur voorkomende substraatlimitaties op genexpressie niveau te bestuderen (**Hoofdstuk 4**). Om het effect van nitraat- of ammoniumlimitatie op anammox bacteriën en autotrofe denitrificeerders te kunnen onderzoeken, hebben wij de eerder beschreven co-cultuur blootgesteld aan substraatlimitatie en de veranderingen in genexpressie (RNAseq) van beide micro-organismen vergeleken met die onder standaard groeiomstandigheden. Tijdens ammoniumlimitatie vertonen 762 genen van de in de co-cultuur aanwezige *Scalindua* sp. meer dan 3 keer hogere of lagere expressie ten opzichte van de standaard conditie. Dit zijn iets meer dan 15% van het totale aantal genen van *Scalindua* sp. Omdat ammonium een belangrijk substraat is, leiden lage concentraties tot een

verlaagde expressie van genen die betrokken zijn bij het energiemetabolisme, transcriptie, translatie, eiwitbiosynthese, celdeling en koolstoffixatie. Bovendien was er onder ammoniumlimitatie een verhoging in de expressie van ammonium transport genen (*amtB*) waar te nemen. De meest significante verhoging in expressie was te zien voor scal00591 (tot 130 keer) en scal00596 (tot 16 keer). Beide genen vormen een cluster met nog twee andere *amtB* genen en twee P-II regulatie genen. De expressie van alle genen binnen dit cluster was minstens 7 keer verhoogd. De twee *focA*-achtige formiaat/nitriet transporter genen die in het *Scalindua* sp. genoom aanwezig zijn, kwamen ook hoger tot expressie onder ammoniumlimitatie en vervingen de *nirC*-achtige transporter (scal00416), het meest getranscribeerde nitriet transporter gen onder standaard condities. Ook genen betrokken bij het aanmaken van een flagel vertoonden hogere expressie onder ammonium limitatie. Nitraatlimitatie had een minder duidelijk effect op *Scalindua* sp. Slechts 228 genen (4.6% van het totaal) hadden een verhoogd of verlaagd expressiepatroon van meer dan 3 keer. Het effect van nitraatlimitatie op *Sedimenticola* sp. was duidelijk sterker, 742 genen vertoonden differentiële regulatie, corresponderend met 17% van het totale aantal genen. De meest significante veranderingen waren de verlaagde expressie van het membraangebonden nitraat reductase complex, en van genen betrokken bij zwavel oxidatie, het conserveren van energie en het centrale metabolisme. Ammonium limitatie had een transcriptie toename van slechts 331 genen (7.5%) tot gevolg, hierbij waren onder andere ammonium transporter genen en genen betrokken in stikstof assimilatie. Dit onderzoek zou kunnen helpen om inzicht te krijgen in de mogelijke rol van genen die coderen voor eiwitten met een onbekende functie. Bovendien geeft deze studie inzicht in de fysiologische rol van bekende eiwitten in anammox bacteriën en *Sedimenticola* sp., door de initiële veranderingen van gen expressie patronen onder invloed van verschillende limitaties te vergelijken.

In **Hoofdstuk 5** werd onderzocht in hoeverre anammox bacteriën in staat zijn om zich aan te passen aan ammonium limitatie door over te schakelen naar een alternatief metabolisme. Aminozuren maken een belangrijk deel uit van het opgeloste, organische stikstof reservoir in mariene ecosystemen en ze zouden als extra elektronen donor kunnen dienen in processen waarin ammonium vrijkomt (denitrificatie of dissimilatieve nitraat reductie naar ammonium (DNRA)). Ze kunnen ook rechtstreeks als ammoniumbron worden gebruikt: De deaminering van aminozuren leidt tot de verwijdering van de aminogroep welke als NH_3 vrijkomt. Om het effect van aminozuren op een anammox verrijkingscultuur te bepalen, werd de anammox bacterie *K. stuttgartiensis* gekweekt in een membraanreactorsysteem onder ammonium

limitatie. Een reactorsysteem werd 1 x daags gevoed met een mengsel van aminozuren (glutamaat, glycine, serine) gedurende een periode van twee weken of continu gevoed met glutamaat gedurende 110 dagen. Veranderingen in de concentraties van de nutriënten werden gemeten en er werd gebruik gemaakt van metagenomics en microscopie om veranderingen van de microbiële gemeenschap in de loop van de tijd te volgen. Het toevoegen van aminozuren stimuleerde de omzetting van nitraat en nitriet en resulteerde in accumulatie van ammonium in de reactor. Als gevolg hiervan werd nitriet limiterend, wat vervolgens leidde tot een competitie voor dit substraat tussen anammox bacteriën en denitrificeerders. Incubatie experimenten met ^{15}N-gelabeld glutamaat of glycine lieten een toename in $^{29}N_2$ zien, wat duidelijk maakt dat aminozuren een bron van ammonium kunnen zijn voor het anammox proces. Gebaseerd op deze resultaten kunnen wij helaas niet concluderen welk lid van de verrijkingscultuur de aminozuren daadwerkelijk omzet. De analyse van het metagenoom toonde aan, dat het toevoegen van aminozuren de groei van denitrificerende en/of fermenterende micro-organismen bevorderde. De microbiële gemeenschap werd, naast *K. stuttgartiensis*, gedomineerd door vertegenwoordigers van de phyla *Proteobacteria* en *Bacteroidetes*. Een stabiele samenwerking tussen heterotrofe denitrificeerders en anammox bacteriën zou belangrijk kunnen zijn in een dynamisch nitraat-rijk ecosysteem waar organische substraten in beperkte mate aanwezig zijn.

Hoofdstuk 6 richt zich op het metabolisme van onvertakte organische vetzuren om zodoende de oxidatie van organische zuren en koolstoffixatieroutes in anammox bacteriën in meer detail te bestuderen. Het AMP-vormende acetyl-CoA synthetase gen (*acs*) van *K. stuttgartiensis*, dat codeert voor een belangrijk enzym betrokken bij de omzetting van organische vetzuren, werd geïdentificeerd en tot expressie gebracht in een *E. coli* gastheer. Op deze manier kon de activatie van een aantal substraten zoals acetaat, propionzuur en boterzuur worden onderzocht. Het meest tot expressie komende acetaat-activerende eiwit (AMP-vormend ACS; kustc1128) in *K. stuttgartiensis* zou een rol kunnen spelen in de Wood–Ljungdahl pathway maar zou ook kunnen leiden tot de directe incorporatie van acetaat door anammox bacteriën. Het *acs* gen codeert voor een eiwit van 589 aminozuren en kon een *E.coli* mutant, die deficiënt was in acetaat-activering, complementeren. Het door een His-tag opgezuiverde eiwit kon acetaat, propionzuur en boterzuur omzetten en had een K_m van 0.19 mM voor acetaat. Deze eigenschappen zouden mixotrofie in anammox bacteriën mogelijk kunnen maken. Naast het *acs* gen, bevatte het genoom van *K. stuttgartiensis* ook de essentiële genen van het acetyl-CoA synthase/CO dehydrogenase complex en twee isoenzymen van aan

Archaea verwante ADP-vormende acetyl-CoA synthetases. Dit geef aan hoe belangrijk acetyl-CoA als tussenproduct in het koolstofmetabolisme van anammox bacteriën is.

Die anaerobe Oxidation von Ammonium (Anammox) ist ein mikrobiologischer Prozess, der durch die Umsetzung von Ammonium und Nitrit zu Stickstoffgas eine wichtige Rolle im globalen Stickstoffzyklus spielt. In natürlichen, aquatischen Ökosystemen sind die Konzentrationen der beiden Anammoxsubstrate limitiert, da NH_4^+ und NO_2^- (Zwischen-) Produkte einiger biogeochemischer Prozesse sind und fortwährend umgesetzt werden. Dementsprechend sind Anammoxbakterien oft abhängig von anderen Mikroorganismen, welche Stickstoffverbindungen umsetzen und NH_4^+ und/ oder NO_2^- freigeben. Dieses Zusammenspiel zwischen Anammoxbakterien und ihrer Umgebung und die verschiedenen Aspekte die hierbei eine Rolle spielen stehen im Mittelpunkt dieser Arbeit. Insbesondere die verschiedenen Prozesse des Stickstoffzyklus, die als Nährstoff- und Substratquellen für Anammoxbakterien dienen können und ihr vielseitiger Metabolismus, der ihnen unter natürlichen Bedingungen von Vorteil sein könnte sind dabei von großem Interesse.

In **Kapitel 2** wurde die Diversität und die Anzahl von Anammoxbakterien in Sedimenten des Guaymas Basin in der Cortés See mit Hilfe anammoxspezifischer Biomarker (die Alpha Untereinheit der Hydrazine Synthase (*hzsA*) und Ladderanlipide) ermittelt. Bohrkerne des Sediments kalter Quellen (cold seeps) und hydrothermaler Felder dienten hierbei als Probenmaterial. Theoretisch würden sich diese Systeme gut als Habitat für Anammoxbakterien eigenen, allerdings gibt es bis dato keine Studien zu ihrem Vorkommen in diesem Gebiet. Alle gewonnenen *hzsA* Amplicons konnten der Gattung '*Candidatus* Scalindua' zugeordnet werden. Die Analyse dieses phylogenetischen Markers deutete auf eine hohe Diversität innerhalb der *Scalindua*-Gattung hin. Durch den Vergleich einzelner Sequenzen konnten wir feststellen, dass die Ähnlichkeit der Nukleotide teilweise weniger als 76% betrug. Die Quantifizierung der Anammoxbakterien erfolgte durch die Amplifikation eines 257 bp Fragmentes in einem qPCR Assay. Aus den Resultaten konnten wir schließen, dass die relative Häufigkeit der Anammoxbakterien in den Sedimenten der kalten Quellen bis zu 90% höher liegt, als in den Hydrothermalfelder und der Referenzzone. Zusätzlich konnten anammoxspezifische Ladderanlipide in einigen Sedimentproben nachgewiesen werden, welche Ergebnisse der *hzsA* Analyse bekräftigten. Da hohe Konzentration reduzierter Schwefelverbindungen in dem untersuchten Ökosystem gemessen wurden, was möglicherweise Einfluss auf den Stickstoffkreislauf haben könnte, wollten wir auch eine Übersicht der wichtigsten Sulfid reduzierenden Mikroorganismen in diesen Systemen bekommen (siehe hierzu auch **Kapitel 3**). Dafür haben wir die Alpha

Untereinheit der Adenosin-5'-phosphosulfat (APS) Reduktase als Phylomarker verwendet. Mithilfe dieses amplifizierten Genes konnten wir überwiegend *aprA* Sequenzen von Sulfid oxidierenden Gammaproteobakterien nachweisen, was auf eine ‚Interaktion' zwischen Anammoxbakterien und Sulfidoxidierern hindeuten könnte. Zusätzlich zeigten einige *aprA* Sequenzen große Übereinstimmungen mit Sulfat reduzierenden Deltaproteobakterien.

In **Kapitel 3** wird das Zusammenspiel zwischen Anammoxbakterien und Sulfid oxidierenden Bakterien näher untersucht. Die Kopplung von Sulfid-abhängiger Denitrifikation und des Anammoxprozesses ist besonders interessant, da Sulfid oft reichlich in Sedimenten vorkommt und -neben Wasserstoff- der wichtigste Reduktor an der Chemokline in anoxischen, marinen Gewässern ist. Zunächst konnten wir zeigen, dass Annammoxaktivität schon bei micromolaren Sulfid-Konzentrationen inhibiert wird. Daraufhin prüften wir, ob der Anammoxprozess mit Sulfid-abhängiger Denitrifikation verbunden werden kann, wenn die *in situ* Konzentration von Sulfid im Reaktor selber unter der Detektionsgrenze (5 μM) bleibt. In einem solchen System würde der denitrifizierende Partner Nitrat, mit Hilfe der Oxidation von Sulfid, zu Nitrit reduzieren, welches dann von Anammoxbakterien gebraucht werden kann. Um diese Hypothese zu testen wurde ein Reaktormodellsystem mit Anammoxbakterien und dem autotrophen, denitrifizierenden Epsilonproteobakterium *Sulfurimonas denitrificans* DSM1251 inokuliert. Mit Hilfe komplementärer Techniken konnten wir zeigen, dass das Gammaproteobakterium *Sedimenticola* sp. die Rolle des Denitrifiziers übernommen hatte. Die stabile Ko-Kultur von Anammoxbakterien und *Sedimenticola* sp. verbrauchte Sulfid, Nitrat, Ammonium und CO_2. Dabei waren Anammoxbakterien verantwortlich für 65-75% des im Reaktorsystem produzierten Stickstoffgases (N_2). Die Kooperation zwischen Anammoxbakterien und Sulfid-abhängiger Denitrifikation könnte vor allem in Ökosystemen, in welchen der Schwefelzyklus aktiv aber die tatsächliche Sulfid-Konzentration nicht messbar ist, eine wichtige Rolle spielen.

Das Reaktormodellsystem erlaubte uns nicht nur die Physiologie von Anammoxbakterien und einem Interaktionspartner zu erforschen, sondern eröffnete uns auch die Möglichkeit die Auswirkung von verschiedenen Substratlimitationen, wie sie auch in der Natur oft vorkommen, auf der Genexpressionsebene zu bestimmen (**Kapitel 4**). Die Transkriptome der Ko-Kultur unter Substratlimitierung wurde mit dem Transkriptom beider Bakterien unter normalen Wachstumsbedingungen verglichen. Ammoniumlimitierung resultierte für das in der Kultur dominante Anammoxbakterium *Scalindua* sp. in der minimal dreifach erhöhten/verminderten Exprimierung von

762 Genen. Dies entspricht etwas mehr als 15% aller dem *Scalindua* sp. Genom zugeschriebenen Gene. Da Ammonium ein sehr wichtiges Substrat für *Scalindua* sp. ist, führten niedrige Ammonium Konzentrationen zu reduzierter Expression von Genen des Energiehaushalts, der Transkription, Proteinbiosynthese, Zellteilung und der Kohlenstoffassimilation. Desweitern führte die Ammoniumlimitierung zu einem signifikanten Anstieg in der Exprimierung von Genen, die für Ammoniumtransporter (*amtB*) codieren. Die beiden *amtB* Gene die den höchsten Anstieg zeigten (scal00591: 130x & scal00596: 16x) befanden sich in einer Gencluster mit noch zwei anderen Ammoniumtransportgenen und zwei P-II Regulationsproteinen. Die Genexpression aller Gene in diesem Cluster war, verglichen mit der unter normalen Wachstumseigenschaften, um das mindestens 7-fache erhöht. Die beiden *focA*-artigen Formiat/Nitrit Transporter wurden unter begrenztem Ammoniumzufluss auch höher exprimiert und lösten somit den *nirC*-artigen Nitrit Transporter (scal00416), welcher unter normalen Wachstumsbedingungen stark exprimiert wurde, als höchst exprimierten Nitrit Transporter ab. Auch die Expression von Genen, die in der Ausbildung des Flagellums involviert sind, war unter Ammmoniumlimitierung stark erhöht. Die Begrenzung des Nitratzuflusses hatte deutlich weniger Auswirkungen auf die Genexpression von *Scalindua* sp., da nur 228 Gene (4.6%) signifikante Unterschiede in ihrer Expression aufwiesen. In *Sedimenticola* sp. hingegen hatte die Limitierung von Nitrat erheblichere Folgen: 742 Gene wurden signifikant anders exprimiert, 17% all im Genom codierten Gene. Vor allem die Gene des membrangebundenen Nitratreduktasekomplexes, Gene des Schwefelmetabolismus, des Energiehaushalts und des zentralen Stoffwechsels sanken im Expressionslevel. Die Limitierung von Ammonium hatte in *Sedimenticola* sp. die differentielle Regulation von nur 331 Genen (7.5%) zur Folge, darunter die erhöhte Exprimierung von Genen die für Ammoniumtransporter- und Stickstoffassimilationsproteine codieren. Diese Studie könnte, durch den Vergleich der Genexpression unter verschiedenen Bedingungen, Aufschluss über die physiologische Rolle noch unbekannter aber auch bekannter Enzyme im Genom beider Mikroorganismen geben. Ob Anammoxbakterien fähig sind limitierte Ammonium-Konzentrationen durch den Wechsel zu alternativen Stoffwechselprozessen zu kompensieren wurde in **Kapitel 5** näher untersucht. Aminosäuren sind ein wichtiger Bestandteil des gelösten, organischen Stickstoffs in marinen Ökosystemen und können als Elektronendonator in der Denitrifikation und der Reduktion von Nitrat zu Ammonium (DNRA) einen Beitrag zu Ammoniumproduktion leisten. Durch Deaminierung können Aminosäuren zusätzlich als eine direkte

Ammoniumquelle dienen. Aufgrund dieser Tatsachen waren wir interessiert welchen Effekt die Zugabe von Aminosäuren auf eine von *Kuenenia stuttgartiensis*-dominierte, in Ammonium limitierte Anreicherungskultur haben würde. Hierfür haben wir Membranreaktoren mit der eben genannten Biomasse zwei verschieden Bedingungen ausgesetzt: Unter pulse-fed Bedingungen wurde ein Gemisch an Aminosäuren (Glutamat, Serin, Glycin) über zwei Wochen einmal täglich zugeführt. Unter kontinuierlichen Bedingungen wurde Glutamat über einen Zeitraum von 110 Tagen permanent in den Reaktor eingespeist. Die Konzentration der verschiedenen Aminosäuren, Nitrat, Nitrit und Ammonium wurden regelmäßig gemessen und mit Hilfe von Metagenomik konnten wir die Veränderung in der mikrobiellen Gemeinschaft verfolgen. Das Zufügen von Aminosäuren führte zu einem Anstieg der Ammonium-Konzentration und der Nitrat- und Nitritreduktionsraten. Dies führte zu einer Verschiebung des Ammonium:Nitritverhältnisses im Reaktor und verursachte Konkurrenz um Nitrit zwischen Anammoxbakterien und denitrifizierenden Mikroorganismen. Experimente mit ^{15}N-Glutamat und -Glycin konnten durch den Anstieg in $^{29}N_2$ zeigen, dass die Aminogruppe dieser Aminosäuren als Ammoniumquelle für Anammoxbakterien diente. Leider konnten wir aus diesen Resultaten nicht schließen, welches Mitglied der mikrobiellen Gemeinschaft im Reaktorsystem die Aminosäuren an sich umzusetzen vermag. Mittels Metagenomik konnten wir aber zeigen, dass das Zufügen von Aminosäuren das Wachstum denitrifizierender und/oder fermentierender Mikroorganismen fördert, wobei *Proteobakterien* und *Bacteroidetes* die dominanten Stämme zu sein scheinen. Zu erwarten ist, dass die stabile Kooperation zwischen Anammoxbakterien und heterotrophen, denitrifizierenden Mikroorganismen vor allem in nitratreichen Systemen mit zyklisch hohen Konzentrationen organischer Substrate wichtig sein könnte.

Um die Oxidation organischer Säuren und die Kohlenstoffassimilation in Anammoxbakterien besser zu verstehen, richtet sich **Kapitel 6** auf die Umsetzung kurzkettiger Carbonsäuren durch *K. stuttgartiensis*. Dafür wurde die AMP-formende Acetyl-CoA Synthetase von *K. stuttgartiensis* identifiziert und das entsprechende Gen (*acs*) in *Escherichia coli* heterolog exprimiert um die Aktivierung von Acetat, Propionat und Butyrat zu testen. Das heterolog exprimierte Enzym konnte eine *E.coli* dreifach Mutante mit Defekt in allen Acetat-Aktivierungswegen komplementieren, und zeigte Aktivität mit den getesteten Carbonsäuren, wobei Acetat am schnellsten umgesetzt werden konnte. Diese Resultate sind im Einklang mit einem potenziell mixotrophen Wachstum von Anammoxbakterien. Neben *acs* waren im Genom von *K. stuttgartiensis* auch die essentiellen Gene des Acetyl-CoA Synthase/CODH

Dehydrogenase Komplexes und Gene welche vermutlich für zwei mit Archaeen verwandte ADP-formende Acetyl-CoA Synthetasen codieren. Dies betont noch einmal die Bedeutung von Acetyl-CoA als Zwischenprodukt der Kohlenstoffassimilation in Anammoxbakterien.

Chapter 1

An introduction to anammox bacteria

The nitrogen cycle

The element Nitrogen (N) is essential for all known forms of life on our planet Earth. It is an important component of DNA, RNA and proteins. The largest pool of nitrogen can be found in the atmosphere as molecular nitrogen (N_2). This form of nitrogen is biologically available only to a relatively small group of microorganisms -diazotrophs-, which are able to convert N_2 into ammonium (NH_4^+) (Fig. 1, 1) (Dixon and Kahn, 2004). Ammonium can then be assimilated into organic matter (Fig. 1, 2a), which ultimately can be mineralized to ammonium (remineralization; Fig. 1, 2b). Under aerobic conditions ammonium can be oxidized in a two-step process (nitrification): First ammonium is converted into nitrite (NO_2^-) by ammonium-oxidizing bacteria or (thaum)archaea (AOB/AOA) (Fig. 1, 3). In the second step nitrite-oxidizing bacteria (NOB) oxidize nitrite further into nitrate (NO_3^-), the most oxidized form of nitrogen (Fig. 1, 4) (Teske et al., 1994; Hovanec et al., 1998; Wuchter et al., 2006).

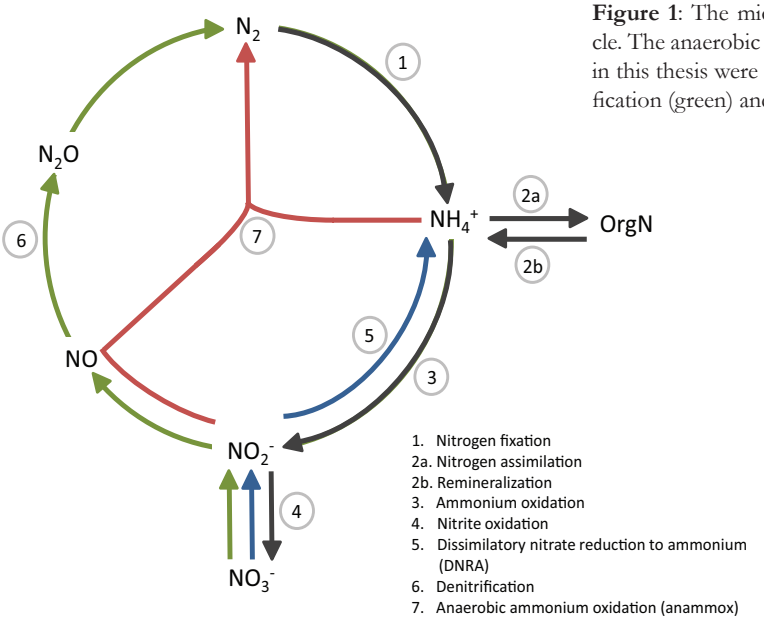

Figure 1: The microbial nitrogen cycle. The anaerobic processes discussed in this thesis were highlighted: denitrification (green) and anammox (red).

In the absence of oxygen, nitrate is thermodynamically the most favorable electron acceptor and there are different pathways in which it can be metabolized. There are a number of nitrate reduction pathways: Dissimilatory nitrate reduction to ammonium (DNRA) (Fig.1, 5) keeps fixed nitrogen in the system, whereas denitrification (Fig.1, 6) and nitrite-dependent anaerobic methane oxidation (Fig.1, 8), coupling the elemental cycles of carbon and nitrogen (Raghoebarsing et al., 2006; Ettwig et al., 2012), result in the release

of fixed nitrogen as gaseous compounds (NO, N_2O, N_2).

In the first step of DNRA nitrate is reduced to nitrite, which is followed by the reduction of nitrite to ammonium. It is assumed that nitrate reduction in organisms performing DNRA proceeds typically via a periplasmic nitrate reductase (*nap*), although there are organisms encoding also or only a membrane-bound nitrate reductase (*nar*) (Potter et al., 2001; Kraft et al., 2011). Nitrite is then converted to ammonium via a pentaheme cytochrome c nitrite reductase system (*nrf*) (Einsle et al., 2001). The electron donor for this reaction can be organic substrates as well as hydrogen, reduced sulfur compounds or methane (Cole, 1988; Dannenberg et al., 1992; Brunet and Garcia-Gil, 1996; Haroon et al., 2013).

Denitrification also starts with the reduction of nitrate to nitrite by a membrane-bound (*nar*) or periplasmic (*nap*) nitrate reductase system. This is followed by the sequential reduction of nitrite to N_2 via NO and N_2O (Fig. 1, 6). Each of the intermediates can also be an end product as the four consecutive steps in denitrification ($NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$) are regulated differentially depending for example on oxygen concentrations and the availability of nitrate/nitrite and electron donor (Betlach and Tiedje, 1981; Hernandez and Rowe, 1987; Härtig et al., 1999), but also on the presence or absence of genes in denitrifying organisms. As a result intermediates, especially the toxic NO_2^- and the potent greenhouse gas N_2O, can escape to the surroundings. The list of organisms able to denitrify is long, ranging from bacteria and archaea to eukaryotes such as fungi and foraminifera (Shoun et al., 1992; Cabello et al., 2004; Risgaard-Petersen et al., 2006). Most denitrifiers are facultative anaerobes, which employ the denitrification pathway only in the absence of oxygen (Zumft, 1997). Two types of denitrification can be distinguished by the use of their carbon source for cell biomass production: Heterotrophic denitrifiers using organic substrates as carbon source and electron donor, have been studied intensively over decades. Autotrophic denitrifiers use inorganic substrates as electron donor i.e. reduced sulfur compounds (HS^-, $S_2O_3^{2-}$, S^0) to reduce nitrate to N_2. They fix CO_2 for cell carbon and the environmental relevance of this process has only been discovered recently (Canfield et al., 2010; Lenk et al., 2011; Bruckner et al., 2013). Autotrophic and heterotrophic denitrification are important in recycling fixed nitrogen in many marine and terrestrial ecosystems.

For a long time denitrification was the only described pathway through which fixed N could be converted into molecular nitrogen. Even though in theory ammonium would also make a suitable electron donor for nitrite or nitrate reduction to N_2 in terms of Gibb's free energy released in the process (Broda,

1977). The fact that ammonium did not accumulate, but in contrast seemed to disappear from anoxic marine water columns had already been observed before Broda's calculations (Richards, 1965; Richards et al., 1971). However it was believed to be due to the assimilation of ammonium as a nitrogen source for denitrification, rather than a biologically mediated process. It was about 20 years later, in the 1990s, that the disappearance of significant amounts of ammonium from a denitrifying fluidized bed reactor treating the effluent stream of a yeast manufacturing company was investigated further (Mulder et al., 1995). Using biomass of this reactor as inoculum an enrichment culture was started. Initially the anaerobic removal of ammonium was thought to be nitrate dependent. It turned out however, that sulfide and/or organic compounds served as electron donors in the system to drive nitrate reduction/denitrification releasing nitrite, which in turn was used by anaerobic ammonium-oxidizing (anammox) bacteria to oxidize ammonium (Mulder et al 1995). Later anammox bacteria were indeed shown to produce N_2 when supplied with NH_4^+ and NO_2^- under anoxic conditions in a one to one ratio (van de Graaf et al., 1996) (Fig. 1, 7). With this discovery research on the 'novel' nitrogen cycle bacteria started to flourish, leading to more understanding but also raising interesting questions regarding their phylogeny, physiology, biochemistry, environmental importance and application after more than 20 years of research as will be outlined in the following sections.

Characteristics of anammox bacteria

Phylogeny

After showing anammox activity the next question that arose was, which microorganisms were responsible for this process? Sequencing the 16S rRNA gene placed the newly discovered anammox bacteria within the phylum of the Planctomycetes (Strous et al., 1999b). They have been assigned to the class of Planctomycetia where they comprise their own order (Candidatus Brocadiales) and family (Candidatus Brocadiaceae) (Jetten et al., 2010). Currently, five genera belonging to the anammox bacteria have been identified (Fig. 2): *Candidatus* 'Brocadia', *Candidatus* 'Jettenia', *Candidatus* 'Anammoxoglobus', *Candidatus* 'Kuenenia' and *Candidatus* 'Scalindua'. Comparing the 16S rRNA genes, the deepest branching genus seems to be *Scalindua*, which predominates marine environments (Kuypers et al., 2003; Hamersley et al., 2007; Woebken et al., 2008; Lam et al., 2009), where the other genera seem to be virtually absent. This indicates a niche differentiation, of which the determining factor is currently still under investigation.

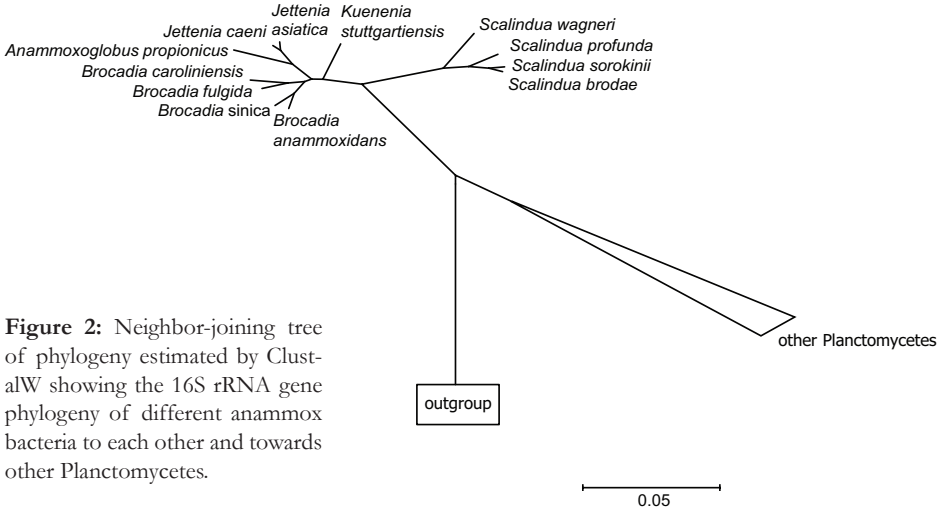

Figure 2: Neighbor-joining tree of phylogeny estimated by ClustalW showing the 16S rRNA gene phylogeny of different anammox bacteria to each other and towards other Planctomycetes.

Catabolism

Anammox is a process that is suggested to be strictly anaerobic. Therefore it is not surprising that anammox bacteria are inhibited by low concentrations of oxygen (Strous et al., 1997, Lam and Kuypers, 2011). This inhibition is reversible as they resume the conversion of substrates as soon as oxygen levels drop below 1 μM. They are slow growers doubling only every 3 to 7 days under our laboratory conditions (Lotti et al., 2014). This requires special biomass-retaining reactor systems to cultivate sufficient biomass (Strous et al., 1998; van der Star et al., 2008; Kartal et al., 2011a). Although they are slow growing, aggregated as well as planktonic anammox biomass seems to have a very low half-saturation constant for both nitrite and ammonium (NH_4^+ K_s = 0.2-0.8 μM; NO_2^- K_s = 3-7 μM determined for planktonic cells), making them very competitive for both substrates (Yan, 2012, Strous et al., 1999a; van der Star et al., 2008). The exact pathway to convert those two substrates into dinitrogen gas has been a conundrum for almost 20 years. From physiological experiments there were indications that hydrazine and/or hydroxylamine could be intermediates (van de Graaf et al., 1997). Biochemical research suggested the involvement of a novel type of hydroxylamine oxidoreductase, typically converting hydroxylamine to nitrite in ammonium oxidizing bacteria, might be involved in the process (Schalk et al., 2000). But it was only after the metagenome of the current model anammox bacterium *Kuenenia stuttgartiensis* became available in 2006, that candidate enzymes could be identified and a theoretical pathway based on *in silico* analysis was proposed (Strous et al., 2006). Combined with acquired biochemical data (Kartal et al., 2011b) there is sufficient evidence for a model in which anammox bacteria

first reduce nitrite to NO (eqn. 1). The enzyme involved in this step seems to differ among anammox genera: Whereas *Kuenenia stuttgartiensis* and *Scalindua profunda* encode a cd_1-type nitrite reductase (*nirS*) (Strous et al., 2006; van de Vossenberg et al., 2013), *Jettenia caeni* and the very closely related to *Jettenia asiatica* (Fig. 2) contain the copper-containing variant (*nirK*) (Hira et al., 2012; Hu et al., 2012). In *Brocadia fulgida* and *Brocadia sinica* a "conventional" *nir*-type nitrite reductase seems to be absent (Gori et al., 2011; Mamoru Oshiki, personal communication) introducing the possibility of a different enzyme fulfilling this function. In the second step NO is condensed with NH_4^+ to form the very powerful reductant hydrazine (N_2H_4) (eqn. 2). As anammox bacteria are so far the only known organisms producing hydrazine, the enzyme performing this reaction is also unique to these microorganisms. The catalytic complex of this hydrazine synthase (HZS) is composed of 2 (*Scalindua* sp.) or 3 subunits *(K. stuttgartiensis),* which are also the most highly expressed mRNAs in all anammox transcriptomes analyzed so far, pointing to its vital role in anammox catabolism (Kartal et al., 2011b; van de Vossenberg et al., 2013). The last step in anammox catabolism is the oxidation of hydrazine to dinitrogen gas (eqn. 3). Hydroxylamine oxidoreductases have been shown previously to convert hydrazine to dinitrogen gas (Nicholas and Jones, 1960; Hooper and Terry, 1977). This would make one or more of the 8 to 10 HAO-like proteins encoded in anammox genomes good candidates for this conversion (Strous et al., 2006). Purification and *in vitro* characterization of several of these proteins from *K. stuttgartiensis* could indeed show that an HAO-like protein was the dedicated hydrazine dehydrogenase (HDH) performing the last step in the anaerobic oxidation of ammonium (Kartal et al., 2011; Maalcke, 2012). All these conversions result in this overall catabolic reaction (eqn. 4):

$NO_2^- + 2H^+ + e^- \rightarrow NO + H_2O$ (eqn. 1)
$NO + NH_4^+ + H^+ + 3\,e^- \rightarrow N_2H_4 + H_2O$ (eqn. 2)
$N_2H_4 \dashrightarrow N_2 + 4H^+ + 4e^-$ (eqn. 3)

$NH_4^+ + NO_2^- \rightarrow N_2 + H_2O$ (eqn. 4)

Cell biology

The energy metabolism in anammox bacteria is closely linked to their unique cell plan. Like other planctomycetes the members of the *Brocadiales* have a complicated cellular organization (Fuerst and Sagulenko, 2011). In anammox bacteria this includes a central compartment, surrounded by a highly curved membrane, the anammoxosome (van Niftrik et al., 2004; van Niftrik et al., 2008). Localization of several key enzymes involved in catabolism and

energy conservation to the anammoxosome gave rise to the analogy that this compartment might act similarly to mitochondria in eukaryotes: The anammox reaction (eqn. 4) would lead to a buildup of a proton gradient over the anammoxosome membrane which could fuel ATP synthesis by ATPases embedded in it (van Niftrik et al., 2008; Jogler, 2014). The composition of the anammox membranes also represents an interesting and unique feature of anammox bacteria as they are built up of so-called ladderane lipids (Damsté et al., 2002; Rattray et al., 2008). These lipids contain up to five linearly concatenated cyclobutane or cyclohexane rings and form dense biomembranes. The exact function of ladderane lipids is currently unknown, but they might play a role in energy conservation by decreasing the passive diffusion of protons across the membrane.

CO_2 fixation

Anammox bacteria are autotrophs and require CO_2 as a carbon source for growth. They encode all the genes necessary to fix CO_2 via the reductive acetyl-CoA (Wood-Ljungdahl) pathway (Figure 3) (Strous et al., 2006). This pathway requires the input of two molecules of CO_2 to make one molecule of acetyl-coenzyme A (CoA) (Ragsdale & Pierce, 2008). The first CO_2 gets reduced by six electrons to a methyl group in the methyl branch, while in the carbonyl branch the second CO_2 first undergoes a reduction to carbon monoxide (CO), followed by the condensation of the methyl group with CO and CoA to make acetyl-CoA.

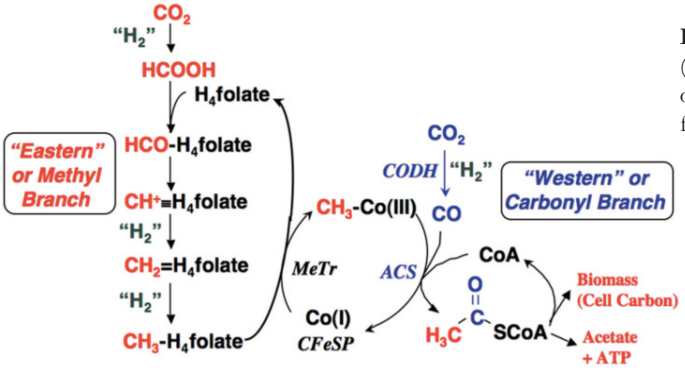

Figure 3: The acetyl-CoA (Wood-Ljungdahl) pathway of carbon fixation (Figure from Ragsdale et al., 2012).

The reaction taking place in the carbonyl branch is catalyzed by a carbon monoxide dehydrogenase/acetyl-CoA synthase complex (CODH/ACS), which has been shown to be active in *K. stuttgartiensis* (Strous et al., 2006). Acetyl-CoA is an important starting point for intermediary metabolism

including the citric acid (TCA) cycle and gluconeogenesis. When anammox bacteria grow autotrophically they accumulate nitrate by oxidizing nitrite. It was previously assumed that this reaction was necessary to gain enough reducing power to perform the initial steps of carbon fixation (Kartal et al., 2012). However, recent evidence suggests that nitrate production does not seem to be directly linked to carbon fixation (Hu et al., 2014). CO_2 fixation might also be mediated by a low-redox potential ferredoxin, which is encoded in an operon together with the catalytic subunits (Strous et al., 2006, Kartal et al., 2013).

Anammox in the environment

Estimating the contribution of the anammox process to nitrogen release on a global scale and assessing distribution and phylogeny of anammox bacteria requires reliable molecular tools. Traditionally the 16S rRNA gene has been a target for amplification in PCR assays and fluorescently labeled probes in fluorescence *in situ* hybridization to detect and quantify anammox bacteria in different environments (Schmid et el., 2001; Kuypers et al., 2003; Schmid et al., 2005; Penton et al., 2006; Humbert et al., 2012). A recurring problem with rRNA gene based molecular techniques to detect anammox bacteria was an either too high or too low specificity of the primers available, making the capturing of anammox diversity and accurate quantification in environmental samples difficult (Schmid et al., 2005; Penton et al., 2006). PCR amplification on functional genes has the advantage that it gives insight into the physiological potential of a microorganism. For anammox bacteria several functional genes have served as biomarkers: Genes encoding hydroxylamine/hydrazine dehydrogenase (HAO/HZO) proteins, the cytochrome cd_1-containing nitrite reductase (*nirS*) and both the alpha and beta subunit of the hydrazine synthase (*hzsA*/*hzsB*) have been used as targets for amplification (Schmid et al., 2008; Lam et al., 2009; Li et al., 2010; Li et al., 2011; Harhangi et al., 2012; Wang et al., 2012). Targeting either *hao* or *nirS* to determine the overall anammox diversity may give rise to a number of problems: (1) All sequenced genomes of anammox bacteria encode at least 8 copies of *hao*-like proteins (Strous et al., 2006; van de Vossenberg et al., 2013; Speth et al., 2015), each of which has homologs in other anammox genera. The high similarity of these homologs makes the interpretation of the data with regard to phylogeny very complex and quantification impossible. (2) As mentioned previously, *nirS* is not present in all anammox genomes making it unsuitable for anammox diversity studies in the environment. A very specific target for detection and diversity studies in environmental samples is the gene cluster encoding the

anammox enzyme hydrazine synthase (*hzsABC*). This cluster is not present in any other prokaryotic and eukaryotic genome sequenced so far, but each anammox genome available contains a single copy. Both the alpha and beta subunit (*hzsA* and *hzsB*) of this gene cluster have been verified as a suitable phylogenetic marker for anammox bacteria in a number of different habitats (Harhangi et al., 2011; Wang et al., 2012).

In several studies ladderane core lipids, which are also a unique feature of the anammox bacteria, have been used as a proxy for the presence of anammox bacteria in environmental samples and geological records (Kuypers et al., 2003; Hamersley et al., 2007; Jaeschke et al., 2009). Four different core lipids have been found to be present in all anammox bacteria analyzed so far: C18-[5]-ladderane fatty acid methyl ester (FAME), C18-[3]-ladderane FAME, C20-[5]-ladderane FAME and C20-[3]-ladderane FAME (Sinninghe Damsté et al., 2005; Rattray et al., 2008; Jaeschke et al., 2009). These results indicate that ladderane analyses can be a very useful complementary tool for PCR-based molecular screening of environmental samples.

Using these different methods anammox bacteria have been detected in virtually any anoxic system containing fixed nitrogen species. They seem to play a central role in the nitrogen cycle in marine systems, especially in oxygen minimum zones (OMZs) and anoxic basins (Kuypers et al., 2003; Kuypers et al., 2005; Woebken et al., 2008; Galán et al., 2009; Lam et al., 2009). But they have also been detected in Artic sediments (Rysgaard et al., 2004; Woebken et al., 2008), river estuaries (Dale et al., 2009), rice paddy fields (Zhu et al., 2011; Hu et al., 2013), peat soils (Hu et al., 2011) and even hot springs (Jaeschke et al., 2009).

Next to molecular tools for detection of anammox bacteria in the environment, determining their activity to assess their contribution to the release of fixed nitrogen is an important point. Commonly, an excess of a ^{15}N-labelled nitrogen species ($^{15}NO_2^-$, $^{15}NO_3^-$ or $^{15}NH_4^+$) is fed to anoxic incubations of an ecosystem of interest. Based on the stoichiometric consumption of nitrite, nitrate and ammonium and the production of $^{29}N_2$ and/or $^{30}N_2$ in differentially labeled incubations, the relative contribution of anammox and denitrification to the N_2 pool can be calculated. Several studies could show that anammox is responsible for 30-70% of N_2 released on a global scale making them essential players in the nitrogen cycle (Kuypers et al., 2003; Dalsgaard et al., 2005; Lam et al., 2009).

In natural aquatic ecosystems anammox bacteria generally occur just below the oxic/anoxic interface, where nitrate starts to decrease and ammonium levels range from micromolar concentrations to non-detectable (Lam and

Kuypers, 2011). As ammonium and nitrite are intermediate products in several biogeochemical processes that are constantly and rapidly turned over, anammox bacteria are dependent on other nitrogen-transforming microorganisms for the supply of their substrates (Figure 4). Ammonium oxidation has been identified as an important source of nitrite. It was suggested that ammonium-oxidizing archaea in the Peruvian oxygen minimum zone could contribute significantly (33%) to the anammox nitrite supply in the suboxic waters at the oxycline (Lam et al., 2009).

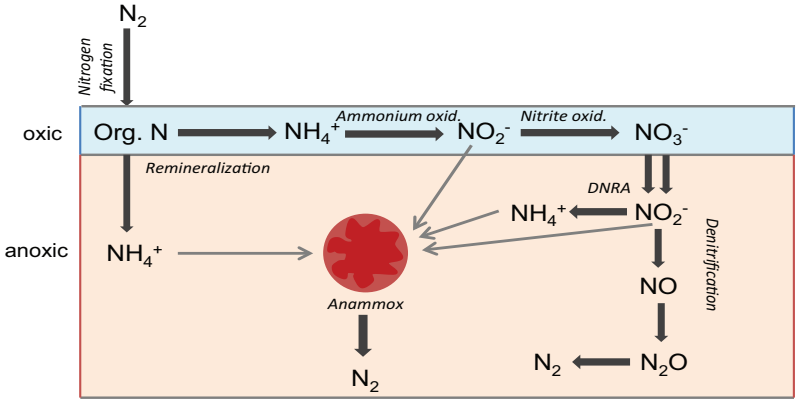

Figure 4: Interaction of anammox bacteria and other N-transforming processes for supply of NH_4^+ and NO_2^-.

Another source of nitrite could be nitrate reduction to nitrite or partial denitrification. This process would require the presence of a suitable electron donor, which might be organic carbon (heterotrophic denitrification) or reduced sulfur compounds (autotrophic denitrification). The fact that sulfide can play an important role in the cycling of nitrogen in marine ecosystems has been shown recently (Canfield et al., 2010): Even though sulfate was continuously reduced to sulfide in the OMZ off the coast of Chile, sulfide concentrations stayed below the detection limit (cryptic sulfur cycling), because sulfide was immediately turned over in the presence of nitrate by autotrophic denitrification. This process has been detected also in other anoxic and nitrate-rich water bodies and might therefore play an important and direct role in the recycling of nitrogen. It might also contribute indirectly to the release of nitrogen by reducing nitrate to nitrite, which in turn could be converted to N_2 by anammox bacteria. These potential sources could release sufficient nitrite to drive the anammox process.

Anammox bacteria also need the second substrate, ammonium, for this reaction. As ammonium and nitrite are converted to dinitrogen gas in a 1:1 ratio

an equal amount of ammonium would be needed. But, the concentrations of ammonium are generally lower right below the oxycline (Lam and Kuypers, 2011), implying that anammox bacteria are probably more often limited in ammonium than in nitrite. One source of ammonium is the remineralization of organic matter, which in the anoxic zone proceeds predominantly via denitrification (Van Mooy et al., 2002). In some ecosystems the rate of anammox activity exceeded denitrification rates however and therefore remineralization was unlikely to account for all of the ammonium supplied to anammox bacteria (Thamdrup et al., 2006; Hamersley et al., 2007; Hannig et al., 2007). A second process that could supply substantial amounts of ammonium and possibly also some nitrite is dissimilatory nitrate reduction to ammonium (DNRA). Its potential importance as a nitrate removal pathway in natural (marine) ecosystems has been unexplored for some time, although recent studies underline its role in the nitrogen cycle in anoxic and sulfidic systems (Brunet and Garcia-Gil, 1996; Burgin and Hamilton, 2007; Dong et al., 2011). Very recently, DNRA could be directly linked to the anammox process in non-sulfidic, open-ocean settings where it could supply substantial amounts of ammonium, which is metabolized quickly by anammox to form N_2 (Kartal et al., 2007; Lam et al., 2009; Jensen et al., 2011). This allows us a glimpse on the complexity of the nitrogen transformation network anammox is part of: A web of interactions of microorganisms from different biogeochemical cycles depending on and competing with each other for substrates in natural (and artificial) ecosystems. Clearly more research is needed to get an idea about the dynamics of such interactions and to identify key players in the different ecosystems.

A versatile metabolism

Surviving in a dynamic environment is increased not only by interaction with other microorganisms, but also by physiological versatility (Atlas et al., 1991). The discovery that anammox bacteria were able to convert formate and acetate to CO_2 while reducing either nitrate or nitrite was the first indication that anammox might have a more versatile metabolism than previously assumed (Güven et al., 2005). This conversion was independent of the addition of ammonium and, if nitrate was added, resulted in a transient accumulation of nitrite. Further studies with purified *Kuenenia stuttgartiensis* cells could finally resolve the fate of nitrate by showing that anammox bacteria were able to perform the dissimilatory reduction of nitrate via nitrite to ammonium at 10% of the normal anammox rate with formate as the electron donor (Kartal et al., 2007). *Brocadia fulgida* and *Anammoxoglobus propionicus* were enriched in

the presence of acetate and propionate respectively and could oxidize these organic acids and out-compete other anammox species and heterotrophic denitrifiers for nitrite (Kartal et al., 2007; Kartal et al., 2008). The main product of organic acid oxidation seems to be CO_2. Despite the fact that formate and acetate could serve as a supplementary carbon source by delivering valuable intermediates in the acetyl-CoA pathway, anammox bacteria seem to continue to fix CO_2 (Güven et al., 2005; Kartal et al., 2007). So far there has been little evidence for mixotrophic growth of anammox bacteria although it would be an advantageous pathway to follow in the presence of organic acids. In theory this might increase the growth rate and/or yield. It is likely that the nitrate-dependent oxidation of formate, acetate and propionate is a common trait in all known anammox bacteria as it has also been observed in *Scalindua profunda* (van de Vossenberg et al., 2008). In addition to short chain organic acids *Kuenenia stuttgartiensis* was also able to convert mono- an dimethylamine and methanol (Kartal et al., 2012, Kartal and de Wild, unpublished data). Looking closer at the genomes of *Kuenenia stuttgartiensis* and *Scalindua profunda*, these anammox bacteria might harbor the potential of converting even more environmentally relevant compounds, i.e. amino acids, hydrogen or Fe^{2+}. The latter has been described as a substrate for nitrate reduction in *Kuenenia stuttgartiensis* and *Brocadia sinica* (Strous et al., 2006; Oshiki et al., 2013). This shows that anammox bacteria might have much more potential for energy conservation to survive in the environment even if ammonium and nitrite are scarce.

Thesis Outline

This thesis aims at gaining a better understanding of the different aspects involved in the interaction of anammox bacteria with other microorganisms under different environmental conditions. The main focus here was the acquisition of nutrients derived from other N-transforming processes and further research was performed on the versatile metabolism of anammox.

In **Chapter 2** we report the diversity and abundance of anammox bacteria in cold seep sediments and hydrothermal vent areas of the Guaymas Basin in the Cortés Sea using unique anammox biomarkers, i.e. hydrazine synthase (*hzsA*) and core ladderane lipids. Due to the high concentrations of sulfide and its potential impact on the cycling of nitrogen as electron donor for nitrate reduction in this ecosystem, we additionally aimed to identify the key players in the oxidation of sulfide in the Guaymas Basin sediments using the alpha subunit of the adenosine-5'-phosphosulfate reductase (*aprA*).

Chapter 3 focusses on the interaction between anammox bacteria and

microorganisms able to oxidize reduced sulfur compounds in more detail. As already micromolar concentrations of sulfide inhibited anammox activity, it was investigated whether the anammox process could be linked to autotrophic denitrification at a continuous but limiting influx of sulfide. We hypothesized that an autotrophic denitrifier would reduce nitrate to nitrite while oxidizing sulfide, allowing anammox to stay active despite an influx of the sulfide. This interaction would not only detoxify sulfide, but also supply anammox bacteria with substrate (NO_2^-). To test this hypothesis a laboratory scale model system containing a co-culture of anammox bacteria and the marine autotrophic denitrifier *Sulfurimonas denitrificans* DSM1251 was started.

Chapter 4 shows that a reactor model system not only allows the study of the physiology of anammox and an interaction partner, but it also represents a convenient way to study the effect of differential limitations of substrates as they might occur in nature on gene expression levels. To elucidate the reaction to nitrate- and ammonium-limiting conditions of anammox and autotrophic denitrifiers we subjected the co-culture described in Chapter 3 to different limitations and the changes in the transcriptome of the key players to the standard growth conditions were compared.

Whether anammox bacteria might be able to overcome ammonium-limiting conditions by using amino acids as alternative electron donor either to reduce nitrate to nitrite and ammonium (DNRA) or by cleaving off the amine group and to combine it with NO_2^- was investigated in **Chapter 5**. To test the effect on anammox cultures, *K. stuttgartiensis* was cultivated under ammonium limitation and either pulse-fed daily with a mixture of amino acids or fed continuously with glutamate for 110 days.

Due to the unexpectedly versatile metabolism anammox bacteria possess, **Chapter 6** focusses on the metabolism of short chain organic acids by functional expression of the most highly expressed acetate-activating enzyme of *K. stuttgartiensis*, an AMP-forming ACS (kustc1128). This enzyme might be involved in the acetyl-CoA pathway, but could also lead to the direct incorporation of acetate by anammox bacteria.

Chapter 2

Presence and diversity of anammox bacteria in cold hydrocarbon-rich seeps and hydrothermal vent sediments of the Guaymas Basin

Russ L., Kartal B., Op den Camp H.J.M., Sollai M., Le Bruchec J, Caprais J.C., Godfroy A., Sinninghe Damsté J.S. and Jetten M.S.M. (2013) Presence and diversity of anammox bacteria in cold hydrocarbon-rich seeps and hydrothermal vent sediments of the Guaymas Basin. *Front Microbiol.* **4:** 219.

Summary

Hydrothermally active sediments are highly productive, chemosynthetic areas which are characterized by the rapid turnover of particulate organic matter under extreme conditions in which ammonia is liberated. These systems might be suitable habitats for anaerobic ammonium oxidizing (anammox) bacteria but this has not been investigated in detail. Here we report the diversity and abundance of anammox bacteria in sediments that seep cold hydrocarbon-rich fluids and hydrothermal vent areas of the Guaymas Basin in the Cortés Sea using the unique functional anammox marker gene, hydrazine synthase (*hzsA*). All clones retrieved were closely associated to the '*Candidatus* Scalindua' genus. Phylogenetic analysis revealed two distinct clusters of *hzsA* sequences (*Ca.* Scalindua *hzsA* cluster I and II). Comparison of individual sequences from both clusters showed that several of these sequences had a similarity as low as 76% on nucleotide level. Based on the analysis of this phylomarker, a very high interspecies diversity within the marine anammox group is apparent. Absolute numbers of anammox bacteria in the sediments samples were determined by amplification of a 257 bp fragment of the *hszA* gene in a qPCR assay. The results indicate that numbers of anammox bacteria are generally higher in cold hydrocarbon-rich sediments compared to the vent areas and the reference zone. Ladderanes, lipids unique to anammox bacteria were also detected in several of the sediment samples corroborating the *hzsA* analysis.

Due to the high concentrations of reduced sulfur compounds and its potential impact on the cycling of nitrogen we aimed to get an indication about the key players in the oxidation of sulfide in the Guaymas Basin sediments using the alpha subunit of the adenosine-5'-phosphosulfate (APS) reductase (*aprA*). Amplification of the *aprA* gene revealed a high number of gammaproteobacterial *aprA* genes covering the two sulfur-oxidizing bacteria *aprA* lineages as well as sulfate-reducers.

Introduction

The Guaymas Basin is a near-shore submarine depression in the central Gulf of California that is characterized by hydrothermally active sediments and hydrocarbon-rich seepages that escape from the sediments at a range of different temperatures (Bazylinski et al. 1988). Geothermally-heated seawater water rises along the ridge segment that is associated with sea floor spreading and escapes via hotspots at high temperatures up to 300°C (Von Damm et al. 1985). Locally, the progressive degradation of organic matter in deeper sediment layers causes the buildup of hydrogen sulfide, methane and other hydrocarbons that seep to the sediment surface at moderate temperatures (cold hydrocarbon-rich seeps) (Vigneron et al. 2013). Due to a high sedimentation rate of the detritus from the productive surface waters as well as terrestrial input, the crustal fissures are covered with a layer of 100-500 m-thick organic-rich sediments (Calvert 1966). The hot fluids diffusing upwards lead to accelerated diagenesis by metal sulfide precipitation and thermochemical decomposition of organic material within the sediment, leading to a distinct seeping fluid with relatively higher concentrations of ammonium and low molecular weight hydrocarbons and lower concentrations of free sulfides than other lava vent sites (Bazylinski et al. 1988; Kawka and Simoneit 1987; Von Damm 1990). This creates an unusual ecosystem at 2,000 m depth that is fueled by conversion of reduced inorganic compounds such as hydrogen sulfide or methane by chemoautotrophs. Although the Guaymas Basin sediment is generally well supplied with ammonium (Von Damm 1990) and there is sufficient evidence of nitrate reduction as a potential source for nitrite (Bowles and Joye 2010; Bowles et al. 2012), anaerobic ammonium oxidation (anammox) has not yet been investigated in Guaymas Basin sediments. It has been assumed that the abundance of reduced carbon and sulfide may favor denitrification and dissimilatory nitrate reduction to ammonium (DNRA) and inhibit anammox (Burgin and Hamilton 2007; Jensen et al. 2008).

Anammox has been shown to be a key process in the cycling of nitrogen in oxygen-limited systems such as oxygen minimum zones and marine sediments all over the world and molecular surveys could confirm the presence of anammox bacteria in very diverse environments such as deep sea sediments, Atlantic hydrothermal vent systems, hot springs, arctic sediments and petroleum reservoirs (Byrne et al. 2008; Harhangi et al. 2012; Hong et al. 2011; Jaeschke et al. 2010; Jaeschke et al. 2009; Kuypers et al. 2005; Kuypers et al. 2003; Lam et al. 2009; Li et al. 2010; Trimmer et al. 2003). So far, anammox bacteria of the genus '*Candidatus* Scalindua spp.' are the major representatives of the order *Brocadiales* in marine ecosystems (Woebken et al.

2008; van de Vossenberg et al. 2008, 2012). Like other anammox bacteria, they derive their energy for growth from the conversion of NH_4^+ and NO_2^- into dinitrogen gas, thereby constituting an important sink for fixed nitrogen under anoxic conditions. In this study we used a combination of specific biomarkers to target anammox bacteria and determine their numbers and diversity within the Guaymas basin. Anammox bacteria are so far the only known bacteria capable of hydrazine production, therefore we used the alpha subunit of the hydrazine synthase (HzsA) complex as a molecular marker to detect and quantify anammox (Harhangi et al. 2012). In addition we used ladderane lipids, which are unique to the membranes of anammox bacteria, as biomarkers. (Sinninghe Damsté et al. 2002, 2005).

The importance of the sulfur cycle in the Guaymas Basin sediments has been known already since the discovery of extensive mats of sulfur-oxidizing *Beggiatoa spp.* that were observed at the sediment interface (Jannasch et al. 1989). These organisms thrive on sulfide and nitrate and contribute significantly to the systems primary production (McHatton et al. 1996; Nelson et al. 1989). As the abundance of reduced sulfur compounds might have an substantial impact on the cycling of nitrogen in the Guaymas Basin sediments, we also investigated the diversity of the gene encoding a key enzyme of the dissimilatory sulfate-reduction pathway: dissimilatory adenosine-5'-phosphosulfate (APS) reductase. Homologues of this gene have been found in photo- and chemotrophic sulfur oxidizers, in which it is thought to work in reverse direction, converting sulfite to APS (Frigaard and Dahl 2009). Two other groups of sulfur oxidizers have been found to be important in marine sediments using different pathways to oxidize reduced sulfur compounds: *Epsilonproteobacteria* using the Sox pathway and *Gammaproteobacteria* related to thiotrophic endosymbionts using the adenosine-5'-phosphosulfate pathway of sulfur oxidation (Hügler et al. 2010). The coupling of the conversion of reduced sulfur compounds to nitrate reduction could have very interesting implications with respect to the formation of complex interactions that would be fueled from the exchange of intermediates.

Results

Biogeochemical analyses

Cold hydrocarbon-rich seep (CS) sediments were generally anoxic (Vigneron et al. 2013) and until a depth of 40 cm the temperature was constant at 3°C. As expected, ammonium was present throughout the sediment in concentrations ranging from 10-40 μM. In one core (1758-14 CT11) ammonium levels dropped below the detection limit (1 μM) at 2-3 cm, but increased again

with increasing depth (Fig. 1A). In sediments with hydrothermal activity ammonium concentrations were higher, increasing from 0.5 mM at the interface to 1.8 mM at 4 cm depth. In deeper layers, concentrations varied between 1.4 and 1.8 mM (Fig. 1A). The temperature at the sediment-water interface was around 20°C and increased linearly to up to 100°C at 40 cm depth.

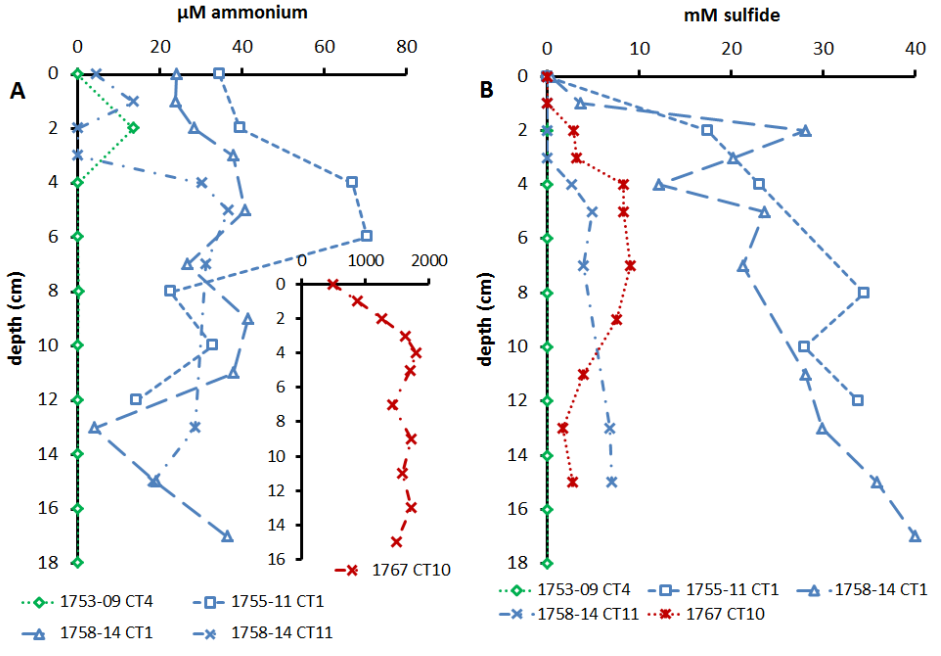

Figure 1: Depth profiles of (A) ammonium and (B) sulfide in cold seep and hydrothermal sediments. Sediment cores of the reference zone is depicted in green, cold hydrocarbon-rich seeps in blue and hydrothermal sites in red. Ammonium in hydrothermal sediments was higher and is therefore shown as an inset (the axes have the corresponding units).

Sulfide was not detected at the sediment interface in any of the samples, but increased rapidly below 1 to 4 cm sediment depth (Figure 1B). In the hydrothermal vent sediment and sediments at the edge of the white mat (1767 CT10 and 1758-14 CT11) concentrations stayed below 10 mM, whereas in sediments visibly covered by mats of sulfur oxidizers (1755-11 CT1 and 1758-14 CT1) sulfide concentrations were higher (Fig. 1B). Within the reference zone (REF) core, which was collected outside of the active zone, there was a small peak of ammonium at 2 cm (13 µM). Sulfide was below detection limit throughout the core. Nitrate and nitrite profiles were not available for the sampling sites.

Ladderane core lipid analysis

To confirm the presence and abundance of anammox bacteria in Guaymas Basin sediments we used ladderane fatty acids as an additional, specific biomarker. Selected samples were analyzed for original ladderane lipids (C_{18} and C_{20} ladderane fatty acids) as well as ladderane oxidation products (C_{14} ladderane fatty acids). The concentration of total ladderanes was highest in the cold hydrocarbon-rich seep sediments (up to 310 ng · g sediment^{-1}) (Tab. 1). The relative contribution of short chain ladderane fatty acids to the total ladderane lipid pool in cold hydrocarbon-rich seep samples was 66% and 74% at the sediment-water interface (samples 1 and 12 respectively) and increased with sediment depth. The reference zone concentrations of ladderane fatty acids were 60 ng · g sediment^{-1} of which 80% were original ladderane lipids. In both hydrothermally active sediments (samples 14 and 15) no ladderane fatty acids could be detected.

Table 1: Concentrations of total ladderane fatty acids in different sediment samples and the relative proportion of short chain ladderane fatty acids.

Sample	1	3	6	9	11	12	13	14	15	17
Ladderane fatty acids (ng· g sediment^{-1})	158	253	234	59	41	157	308	0	0	60
% short chain ladderane fatty acids	74	84	86	86	45	66	75	-	-	20

Diversity of the hzsA gene in Guaymas Basin sediments

Amplifying the *hzsA* gene with two different primer sets (757F$_{Scal}$ / 1857R and 526F / 1857R) resulted in 80 clones from Guaymas Basin sediments, all of which were related to the '*Ca.* Scalindua' genus (Figure 2). Phylogenetic analysis revealed two distinct clusters of *hzsA* sequences ('*Ca.* Scalindua' *hzsA* cluster I and II).

Sequences of cluster II were preferentially amplified using 526F / 1857R as primers during amplification. The average similarity within these clusters was 91.6% for cluster I and 88.7% for cluster II. However, comparison of individual sequences revealed that between both clusters several sequences share a similarity as low as 76% at the nucleotide level. Within the '*Ca.* Scalindua' *hzsA* cluster I three subclusters could be identified: BIG I and BIG II consisted mostly of cold hydrocarbon-rich seep sequences. The third cluster consisted of sequences that were related to the enrichment culture species *S. profunda* (van de Vossenberg et al. 2008) as well as three clones from Northeast Greenland marine sediment (Harhangi et al. 2012). The '*Ca.* Scalindua' *hzsA* cluster II was generally more diverse and composed of two subclusters. The first subcluster containing one sequence from Barents Sea

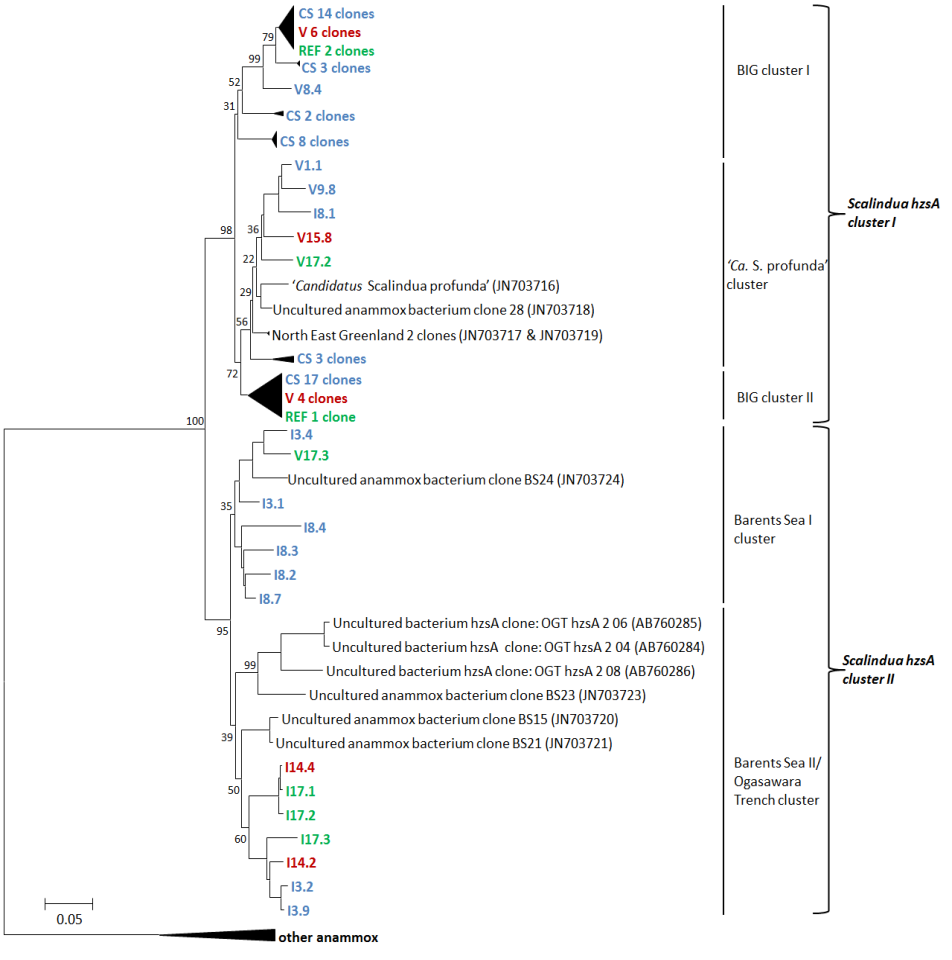

Figure 2: Neighbor-joining tree of phylogeny estimated by ClustalW included in the MEGA 5.0 software package, showing >1000 bp fragments of *hzsA* nucleotide sequences retrieved from the Guaymas Basin sediments. Letters (I or V) of the samples indicate the primer set used for amplification and the number refers to the sample. Samples are color-coded and at collapsed nodes abbreviated: reference zone in green (REF), cold hydrocarbon-rich seeps in blue (CS) and hydrothermal sites in red (V). Values at the internal nodes indicate bootstrap values based on 500 iterations. The bar indicates 5% sequence divergence. The outgroup with other anammox bacteria includes Genbank accession numbers JN703715, JN703714, JN703713, JN703712, AB365070 and CT573071.

sediment and mostly sequences associated with cold hydrocarbon-rich seeps. The second cluster contained several clones retrieved from the Barents Sea (Harhangi et al. 2012), clones from the Ogasawara Trench in the West Pacific (Nunoura et. al 2013) and clones from cold hydrocarbon-rich seeps, vents and reference zone samples. Generally there seems to be no great correlation between the sample locations and the diversity of anammox bacteria as clones retrieved from cold hydrocarbon-rich seep, reference zone and hydrothermal sediments were found in all clusters.

Quantification of anammox bacteria by function gene amplification

Based on the *hzs*A biodiversity study (see above) which showed only representatives of the '*Ca.* Scalindua' genus, we determined absolute numbers of anammox bacteria in the sediment samples by amplification of a '*Ca.* Scalindua'-specific 257 bp fragment of the *hszA* gene in a qPCR assay. The results indicated that numbers of anammox bacteria were generally higher in cold hydrocarbon-rich seep environments compared to the vents (Fig. 3). Gene copy numbers of anammox *hzsA* in cold hydrocarbon-rich seep sediments varied between 1.5 x 10^6 to 1.5 x 10^7 copies per gram of sediment. The total number of *hzsA* gene copies was usually highest at the sediment interface and decreased with increasing depth (Fig.3, 9/10, 12/13, 15/16). The exception was a whole sediment core (Fig. 3, 1-7) (0-21 cm), which was analyzed in increments of 3 cm. Here, *hzsA* gene copies increased linearly from the interface peaking at 6-9 cm sediment depth (1.47 x 10^7 copies · g sediment^{-1}). In proximity of the hydrothermal vents total numbers of the *hzsA* gene in the sediments were much lower (1.9 x 10^5 to 1.3 x 10^6 copies · g sediment^{-1}).

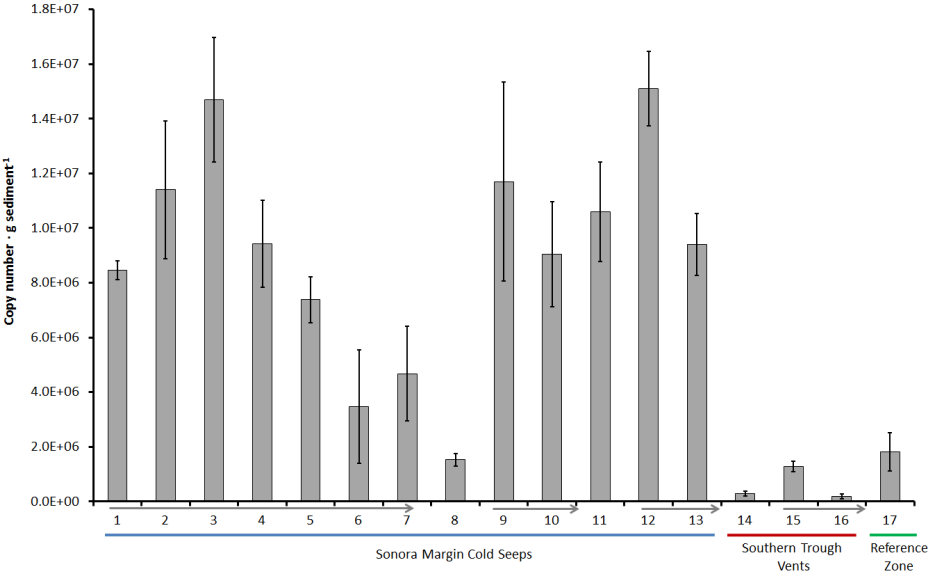

Figure 3: *hzsA* copy number in different sediment samples (±SD of technical replicates). Sediment cores of the reference zone is depicted in green, cold hydrocarbon-rich seeps in blue and hydrothermal sites in red. Arrows indicate decreasing sediment depth within a single core.

Diversity of the aprA gene

An *aprA* library comprising 39 sequences was generated from selected cold hydrocarbon-rich seep sediments (samples 1,3 and 9) and the sediment interface

of the vents MegaMat M27 (sample 15). The majority of all sequences were affiliated with the *Gammaproteobacteria* (35 sequences), clustering in both *apr* lineages of known sulfur oxidizing bacteria (SOB) (Meyer and Kuever 2007). Sequences from lineage I were divided into 2 clusters (Fig. 4). The first cluster consisting of 4 cold hydrocarbon-rich seep clones and 5 from hydrothermal vent sediments were most closely related to gut microflora clones of *Asterechinus elegans* (93%) and endosymbionts of *Olavius ilvae* (86-89%) (Becker et al. 2009; Ruehland et al. 2008). The second cluster was comprised of 11 cold hydrocarbon-rich seep clones and 3 vent clones showing the highest similarity to clones retrieved from hydrothermal vents of the Logatchev field (87-89%) and low temperature hydrothermal oxides at South West Indian ridge (90%) (Hügler et al. 2010). One clone was retrieved within lineage I being most closely related with the uncultured alphaproteobacterial *aprA* genes.

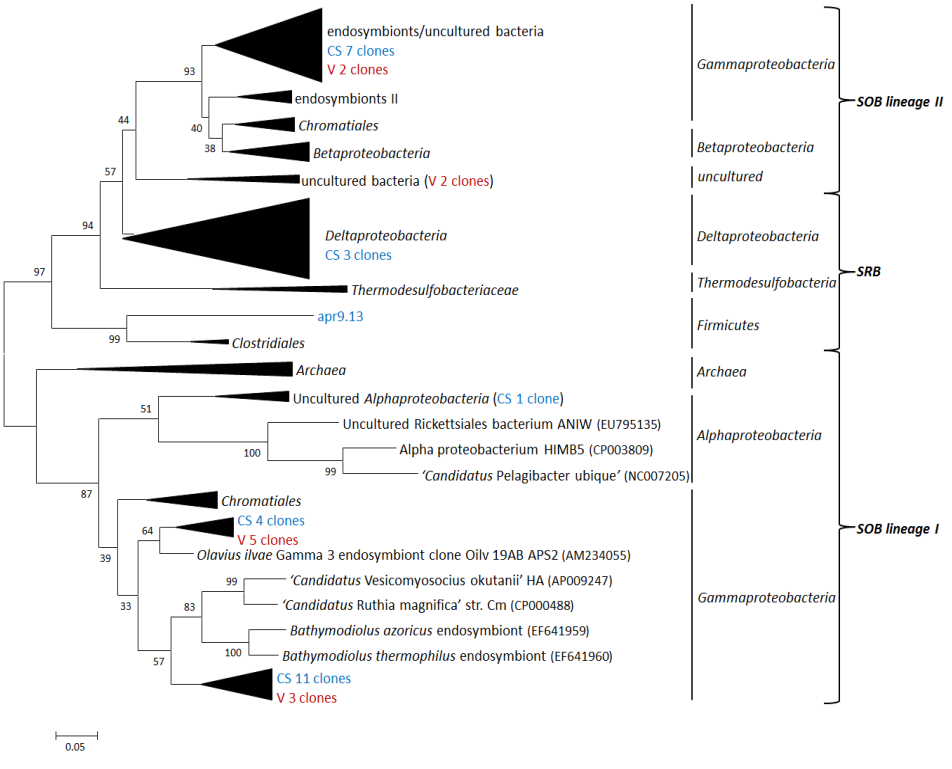

Figure 4: Neighbor-joining tree of phylogeny estimated by ClustalW included in the MEGA 5.0 software package, showing ~400 bp fragments of *aprA* nucleotide sequences retrieved from the Guaymas Basin sediments. The number refers to the sample and collapsed nodes are abbreviated: reference zone in green (REF), cold hydrocarbon-rich seeps in blue (CS) and hydrothermal sites in red (V). Values at the internal nodes indicate bootstrap values based on 500 iterations. The bar indicates 5% sequence divergence.

The closest hit was a clone retrieved from carbonate sediments at the South West Indian Ridge (88%). Within SOB *apr* lineage II 9 sequences (7 CS and 2 V) grouped within a cluster of endosymbionts and uncultured bacteria of deep-sea environments. Closest hits were obtained with marine sediment clones of the Cascadia margin (94%), gut microflora clones of *Asterechinus elegans* (92%) and endosymbionts of *Riftia pachyptila* (86%) (Becker et al. 2009; Blazejak and Schippers 2011; Brissac et al. 2011; Lenk et al. 2011; Meyer and Kuever 2007). Two clones from vent sediments formed a separate cluster in *apr* lineage I, showing a rather low similarity to previously described clones (gut microflora clones of *Asterechinus elegans* 81%). Three of the *aprA* sequences clustered with sulfate-reducing *Deltaproteobacteria* of the *Desulfovibrio* and *Desulfobulbus* genus and a single sequence was most closely related with the firmicutes (*Desulfotomaculum sp.* 78%) (Friedrich 2002; Hügler et al. 2010).

Discussion

Almost all samples from the cold hydrocarbon-rich seep sediment core that were investigated contained a high relative abundance of short chain ladderane fatty acids (> 65%). This high percentage may be explained by degradation of original ladderanes during diagenesis (Rush et al. 2012). Little is known about the biological degradation of ladderanes yet, but it is assumed that it proceeds via the β- oxidation pathway (Beam and Perry 1974; Dutta and Harayama 2001). Although the sediments were virtually anoxic, the degradation of original ladderane fatty acids could be the result of periodic exposure to low amounts of oxygen in such a dynamic system. When the concentrations of short and original ladderane fatty acids were compared, a slight increase of original ladderanes and an increase in short chain ladderanes with depth was observed, which corresponded to the acquired qPCR data of core 1758-14 CT2 (Fig. 5). This would suggest that on the one hand there was ongoing conversion from long chain fatty acids to the short chain ladderanes in the absence of oxygen, but also that the overall concentration of original ladderanes was higher deeper in the sediment at this specific location. This could be the result of seasonal high burial of anammox biomass and a high ladderane turnover or the *in situ* production of ladderanes in deeper sediment layers. In hydrothermally active sediments no ladderanes could be detected. This could be because ladderanes were unstable at higher temperatures due to their peculiar chemical structure (Jaeschke et al. 2008).

The use of two different primer sets to amplify *hzsA* gene fragments resulted in the retrieval of marine *hzsA* sequences showing a diversity comparable to that reported for 16S rRNA genes of anammox bacteria (Woebken et al.

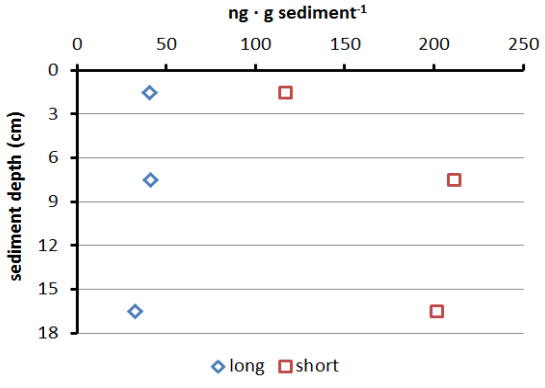

Figure 5: Depth profile of absolute ladderane lipid concentrations in core 1758-14 CT2 including original ladderanes and short chain ladderane fatty acids. Data points represent measurements on the sediment sample pooled from 0-3cm, 6-9cm and 15-18cm sediment depth.

2008). The functional gene analysis hinted at the high diversity within the '*Ca.* Scalindua' genus, considering that some genera of fresh water anammox shared a higher similarity (*Ca.* Jettenia asiatica and *Ca.* Brocadia anammoxidans 79.4%). This was a first indication using a functional gene that the marine group might consist of more than one genus or at least showed a very high interspecies diversity within a single genus.

The finding of anammox specific phylomarkers within these sediments was surprising, as the deep sea sediments of the Guaymas Basin were not only rich in organic matter, ammonium and methane, but also contained mM concentrations of sulfide. Sulfide has been reported to inhibit the anammox process in granular sludge of wastewater treatment plants already at low concentrations (Carvajal-Arroyo et al. 2013; Jin et al. 2013). Although no conclusion can be made with regard to anammox activity in the Guaymas Basin sediments, the results point to the same direction as the highest number of *hzsA* copy numbers usually coincided with the absence of sulfide at the sediment interface and decreased rapidly with depth.

Aerobic ammonium oxidizing Thaumarchaeota were shown to play a role in supplying nitrite for anammox in oxygen minimum zones (OMZ) (Francis et al. 2005; Lam et al. 2009), but in these systems also a cryptic sulfur cycle was reported (Canfield et al. 2010). Since for continental shelf sediments and the Benguela OMZ denitrification was suggested to play a role in nitrite supply (Thamdrup and Dalsgaard 2002; Kuypers et al. 2005) and the sites of our study are all sulfidic in nature we focused on the possibility of sulfide-driven partial denitrification. Reduced sulfur compounds, such as sulfide, fuel primary production in cold hydrocarbon-rich seeps and are often linked to oxygen and nitrate respiration (Jannasch and Wirsen 1979; Karl et al. 1980; Lichtschlag et al. 2010). The amplification of a key gene in sulfur oxidation revealed a high number of gammaproteobacterial *aprA* genes covering the two SOB *aprA* lineages as well as sulfate-reducers. Previously also *Epsilonproteobacteria*

were shown to be abundant in Guaymas Basin sediments likely also gaining energy from growth on sulfide as an electron donor to reduce nitrate (Bowles et al. 2012; Teske et al. 2002). This suggested that reduced sulfur compounds could serve as a link between sulfur and nitrogen cycling in such ecosystems. Although there was no direct evidence for the significance of *Gammaproteobacteria* in sulfur oxidation in the Guaymas Basin sediments the retrieval of such an *aprA* diversity confirmed findings of *Gammaproteobacteria* playing a role in linking the sulfur and nitrogen cycles in marine sediments by coupling sulfide oxidation to nitrate reduction (Hügler et al. 2010; Lenk et al. 2011; Mills et al. 2004). This could have very interesting implications with respect to the formation of complex interactions that would be driven by the exchange of intermediates (i.e. nitrite). For example, partial denitrification (Błaszczyk 1992, unpublished data) coupled to sulfide oxidation could supply anammox bacteria with nitrite. Additionally, the oxidation of sulfide might create pockets in which the concentration of free sulfide is low enough so that anammox bacteria remain active. The existence of such an interaction was recently reported (Wenk et al. 2013), but whether this could occur in the sediments or the water column of the Guaymas Basin remains to be determined.

Conclusion

This study shows that anammox bacteria were detected in complex and exotic environments by amplification of the unique functional marker gene *hzsA*, allowing much more specificity than 16S rRNA gene based analysis. The high diversity observed in *hzsA* phylogeny suggested a high interspecies variety within the marine anammox cluster in an essential and highly-conserved gene. Although evidence so far did not favor anammox bacteria in sulfidic sediments (Burgin and Hamilton 2007) we detected relatively high numbers of anammox gene copies in cold hydrocarbon-rich seep sediments of the Guaymas Basin.

Experimental procedures

Sample collection

Samples were recovered from cold hydrocarbon-rich seeps and hydrothermal vent sediments during the cruise "BIG" (RV L'Atalante, June 2010) on dives 1758-14, 1755-11, 1764-20 and 1766-22 as described by Vigneron et al. (2013). Sets of one location were sampled at about 12 cm distance. A description of the samples can be found in Tab. 2.

Table 2: Description of the different samples used in this study.

No	Dive	Core	(cm)	Location	Zone	Date	depth (m)	Latitude	Longitude	Reference
1	1758-14	CT2	0-3							
2	1758-14	CT2	3-6							
3	1758-14	CT2	6-9	Cold Seep,						
4	1758-14	CT2	9-12	Vasconcelos active site,	Sonora Margin	6/19/2010	1574	N 27 35.5750	W 111 28.9840	Vigneron *et al*. 2013
5	1758-14	CT2	12-15	white mat.						
6	1758-14	CT2	15-18							
7	1758-14	CT2	18-21							
8	1758-14	CT1		Cold Seep, Vasconcelos active site, white mat.	Sonora Margin	6/19/2010	1574	N 27 35.5754	W 111 28.9860	
9	1758-14	CT11	0-4	Cold Seep,						
10	1758-14	CT11	4-6	Vasconcelos active site, edge of white mat.	Sonora Margin	6/19/2010	1574	N 27 35.5872	W 111 28.9859	
11	1755-11	CT1		Cold seep, Vasconcelos active site.	Sonora Margin	6/16/2010	1573	N 27 35.5827	W 111 28.9848	
12	1755-11	CT2	1-1.5	Cold seep,						
13	1755-11	CT2	6-7	Vasconcelos active site.	Sonora Margin	6/16/2010	1573	N 27 35.5820	W 111 28.9832	
14	1764-20	CT3	0-2	Hydrothermal vent, Mat Mound active site, orange mat.	Southern Trough	6/27/2010	2005	N 27 00.3772	W 111 24.5641	Callac *et al*. 2013
15	1766-22	CT2	0-5	Hydrothermal vent,						
16			5-10.5	MegaMat M27 active site, white mat.	Southern Trough	6/29/2010	2003	N 27 00.4461	W 111 24.5243	
17	1753-09	CT4	8-11.5	Reference zone		6/14/2010	1850	N 27 25.4835	W 111 30.0779	Vigneron *et al*. 2013

Chemical analyses

Pore water from the cores was extracted in one (< 10 cm) or two centimeters (> 10 cm) resolution using Rhizon moisture samplers with a pore size of 0.1 µm (Seeberg-Elverfeldt et al. 2005). Water samples were subsampled by adding either $ZnCl_2$ (1:1 vol/vol) for H_2S analysis or freezing at – 20°C (ammonium) until further analysis. Ammonium was analysed by a manual flourimetric method (detection limit 1 µM, Holmes et al. 1999). This analysis is sensitive to the presence of hydrogen sulfide (H_2S) therefore the measurements were corrected for the presence of H_2S. Hydrogen sulfide concentrations were determined by colorimetry according to Fonselius et al. (2007).

DNA isolation and polymerase chain reaction

Total genomic DNA was isolated from different sediments using the PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Inc.) according to the manufacturer's protocol. To avoid contamination with our own anammox cultures, isolations were performed in a different department.

All PCR amplifications were performed in a total volume of 25 µl using 12.5 µl PerfeCTa® SYBR® Green FastMix (Quanta), 0.4 µM forward/reverse primer, 2 µl of template and 10.5 µl of DEPC-treated H_2O. Hydrazine synthase amplification was initiated with a denaturation step at 94 °C for 5 min and continued with a standard amplification program of 35 cycles (45 sec 94 °C; 1 min 56 °C; 45 sec 72 °C). The final elongation step was done at 72 °C for 7 min. Two different primer combinations, targeting the single copy *hzs*A gene, were used on the different samples (Harhangi et al. 2012): I (526F-TAYTTTGAAGGDGACTGG; 1857R-AAABGGYGAATCATARTGGC) and V ($757F_{ScaI}$-AGTTCNAAYTTTGACCC; 1857R-AAABGGYGAATCATARTGGC). The *aprA* fragments of 387 bp were amplified using primers aprA-1-FW-TGGCAGATCATGATYMAYGG and aprA-RV-GGGCCGTAACCGTCCTTGAA in the same assay as described for hydrazine synthase (Meyer and Kuever 2007). The annealing temperature was lowered to 55 °C.

Cloning and phylogenetic analysis

The *hzsA* and *aprA* amplicons were cloned in *Escherichia coli* using the pGEM T-easy vector system (Promega) according to the protocol. Clones were randomly selected from overnight-grown LB agar plates containing 100 µM of ampicillin, 200 µM IPTG and 200 µM X-gal. Plasmids were isolated with the GeneJet Plasmid Miniprep Kit (Thermo Fisher Scientific) according

to the protocol using 4 ml of overnight bacterial culture. Colony PCR was performed to check cloned plasmids for an insert. 80 *hzsA* clones and 40 *aprA* clones were selected for Sanger sequencing at the DNA Diagnostics Department of Nijmegen University Medical Center, Nijmegen. Alignments and phylogenetic analysis were performed using the MEGA 5.0 software (Tamura et al. 2011). Related sequences were retrieved via BLAST searches in the Genbank databases. The sequences were submitted to Genbank under the accession numbers KF202916-KF202955 for *aprA* and KF202956-KF203035 for *hzsA*.

Quantitative PCR

Primers targeting the *hzsA* gene (1600F$_{Scal}$-GGKTATCARTATGTAGAAG; 1857R-AAABGGYGAATCATARTGGC) were used in a quantitative PCR assay to assess the absolute number of anammox bacteria in the deep sea samples. Amplifications were again performed in a total volume of 25 µl using iQ™ SYBR® Green Supermix (Bio Rad) and 1 µl template. The amplicon of '*Ca.* Scalindua profunda' with a fragment amplified with the same primers (van de Vossenberg et al. 2012) was diluted in tenfold steps and used as a standard in the analysis. Amplification was done on a iCycler iQ (Bio Rad) according to the following thermal protocol: The amplification program was started with 3 min at 96°C, followed by 40 cycles of 1 min at 95°C, 1 min 54°C, and 1 min at 72°C and a final elongation step of 5 min at 72°C. A melting curve analysis was performed at the end of the program ranging from 52 - 90°C in steps of 0.5°C to identify potentially unwanted amplicons. Ten products that were retrieved were cloned into *E. coli* as described above to verify amplification of the correct product. Retrieved plasmids were checked for an insert by colony PCR and sequenced as described before.

Lipid analysis

Ladderane fatty acids, including the newly identified short-chain C_{14} ladderane fatty acids, were analyzed according to previously described methods (Hopmans et al. 2006; Rush et al. 2012). Briefly, sediments were freeze-dried, homogenized and extracted using a modified Bligh-Dyer method (Boumann et al. 2006). The extract was saponified by refluxing with aqueous 1 N KOH in 96% methanol for 1 h. The pH of the saponified extract was adjusted to 3 with 2 N HCl in methanol and the fatty acids were extracted with dichloromethane (DCM). The DCM fraction was dried using Na_2SO_4 and the fatty acids were converted into their corresponding fatty acid methyl esters (FAMEs) by methylation with diazomethane (CH_2NH_2). A

FAME fraction was obtained by elution over activated aluminum oxide with DCM. Polyunsaturated fatty acids (PUFAs) were removed by elution over a small AgNO3 (5%)-impregnated silica column with DCM. The resulting fraction was dissolved in acetone (1 mg/ml) and filtered through a 0.45 μm, 4 mm diameter polytetrafluoroethylene (PTFE) filter and analyzed by high performance liquid chromatography coupled to positive-ion atmospheric pressure chemical ionization tandem mass spectrometry (HPLC/APCI-MS/MS) in selected reaction monitoring (SRM) mode as described by Rush et al. (2012). Ladderane FAMEs were quantified using external calibration curves of isolated methylated ladderane fatty acid standards.

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Chapter 2

Chapter 3

Interactions between anaerobic ammonium and sulfur-oxidizing bacteria in a laboratory scale model system

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Summary

Fixed nitrogen is released by anaerobic ammonium oxidation (anammox) and/or denitrification from (marine) ecosystems. Nitrite, the terminal electron acceptor of the anammox process, occurs in nature at very low concentrations, and is produced via (micro)aerobic oxidation of ammonium or nitrate reduction. The coupling of sulfide-dependent denitrification to anammox is particularly interesting because besides hydrogen, sulfide is the most important reductant at the chemocline of anoxic marine basins and is abundant within sediments. Although at μM concentrations sulfide may be toxic and inhibiting anammox activity, a denitrifying microorganism could convert sulfide and nitrate at sufficiently high rates to allow anammox bacteria to stay active despite an influx of sulfide. To test this hypothesis a laboratory scale model system containing a co-culture of anammox bacteria and the autotrophic denitrifier *Sulfurimonas denitrificans* DSM1251 was started. Complementary techniques revealed that the gammaproteobacterial *Sedimenticola* sp. took over the intended role of *Sulfurimonas denitrificans*. A stable coculture of anammox bacteria and *Sedimenticola* sp. consumed sulfide, nitrate, ammonium and CO_2. Anammox bacteria contributed 65-75% to the nitrogen loss from the reactor. The cooperation between anammox and sulfide-dependent denitrification may play a significant role in environments where sulfur cycling is active and where actual sulfide concentrations stay below μM range.

Introduction

The suboxic regions in the oceans, such as continental shelf sediments, areas of limited circulation (i.e. fjords or basins) and oxygen minimum zones (OMZs) are major sinks for fixed nitrogen in nature (Falkowski, 1997; Gruber and Galloway, 2008). Denitrification and anaerobic ammonium oxidation (anammox) are the two main pathways that use inorganic nitrogen and release dinitrogen gas (N_2) (Lam and Kuypers, 2011). Anammox bacteria derive their energy for growth from the reduction of nitrite (NO_2^-), combining it with ammonium (NH_4^+) into N_2, thereby constituting a sink for fixed nitrogen. Their contribution to nitrogen loss from several anoxic marine systems has been estimated to be more than 50% (Dalsgaard *et al.*, 2003; Kuypers *et al.*, 2003; Arrigo, 2005; Lam *et al.*, 2009). Ammonium and nitrite are intermediate products in several biogeochemical processes and they are constantly and rapidly turned over at oxic/anoxic interfaces via ammonium oxidation and nitrite oxidation in the presence of O_2 (Füssel *et al.*, 2012) or by denitrification and/or anammox in the absence of O_2 (Francis *et al.*, 2005; Lam and Kuypers, 2011; Zehr and Kudela, 2011). This means that nitrogen-transforming microorganisms have to compete with and are dependent on each other for the supply of their substrates.

It was estimated that ammonium-oxidizing archaea (AOA) could supply substantial amounts of nitrite (33%) in the suboxic waters at the oxycline in the Peruvian oxygen minimum zone (OMZ) (Francis *et al.*, 2005; Lam *et al.*, 2009). This was also tested in a laboratory-scale model system, which showed that AOA could indeed provide nitrite for anammox bacteria under oxygen limitation (Yan *et al.*, 2012). An alternative source of nitrite for anammox is nitrate reduction to nitrite. The peak in anammox cell numbers and their high activity at zones of nitrate decrease already points to a co-occurrence within the same niche (Kuypers *et al.*, 2005; Hamersley *et al.*, 2007; Galán *et al.*, 2009). The fact that nitrate is indeed a major source of nitrite for anaerobic ammonium oxidation was shown by labeling experiments in continental shelf sediments as well as in the Benguela and Chile OMZs, where massive losses of nitrogen by coupling partial denitrification to anammox were observed (Thamdrup and Dalsgaard, 2002; Kuypers *et al.*, 2005; Canfield *et al.*, 2010). Ammonium and nitrite may also be produced through dissimilatory nitrate reduction to ammonium (DNRA). Recent studies highlighted the importance of this relatively overlooked process that could contribute significantly to the cycling of nitrogen in benthic systems, at hydrothermal vent sites, but also open waters systems (Preisler et al., 2007; Lam et al., 2009; Dong et al., 2011; Kamp et al., 2011). The potential coupling of DNRA and anammox would

result in the production of double labeled $^{15}N^{15}N$ by anammox bacteria, thereby interfering with traditional isotope pairing experiments, in which the production of $^{30}N_2$ from ^{15}N nitrate is used as a proxy for denitrification (Kartal *et al.* 2007).

Nitrate reduction via denitrification and DNRA require the presence of a suitable electron donor, which in the case of heterotrophic denitrification is organic carbon. There are also several sulfur-oxidizing bacteria that are able to reduce nitrate using inorganic, reduced sulfur compounds (i.e. sulfide, thiosulfate, etc.) as electron donors (reviewed in Shao *et al.*, 2010). This so-called autotrophic denitrification is performed by several representatives of the Alpha, Beta-, Gamma- and Epsilonproteobacteria. Indications of the importance of this process were observed in the stratified water column of the Black Sea (Vetriani *et al.*, 2003), the Baltic Sea (Labrenz *et al.*, 2005; Glaubitz *et al.*, 2009), the upwelling systems off the coast of Chile (Canfield *et al.*, 2010) and Namibia (Lavik *et al.*, 2009), where especially representatives of the Gamma- and Epsilonproteobacteria seemed to play an important role in dark carbon fixation and sulfide detoxification. The exact mechanism favoring either anammox or denitrification is still unknown. However, as autotrophic denitrification uses sulfide as electron donor and there are indications that anammox activity is inhibited by μM levels of sulfide, such an interaction is unlikely to occur in sulfidic waters (Dalsgaard *et al.*, 2003; Jensen *et al.*, 2008; Jin *et al.*, 2013).

Nevertheless, anammox bacteria were discovered in a denitrifying fluidized bed reactor, where anaerobic ammonium removal was dependent on nitrate with sulfide and/or organic compounds being the major electron donors in the system (Mulder *et al.*, 1995). This already indicated that sulfide-based partial denitrification was probably feeding anammox with nitrite. Furthermore, anammox activity was stimulated when sulfide was present accompanied with a transient accumulation of nitrite, probably also the result of partial, autotrophic denitrification (van de Graaf *et al.*, 1996).

Such an interaction of autotrophic sulfide oxidizing denitrifiers and anammox bacteria could also occur in nature. Indeed, the discovery of a cryptic sulfur cycle within the OMZ of Chile in the absence of measureable concentrations of sulfide revealed the possibility for nitrate reduction to nitrite coupled to the oxidation of sulfide to supply anammox with considerable amounts of nitrite in these environments (Canfield *et al.*, 2010). Due to the high turnover of sulfide to sulfate and accordingly nitrate to nitrite by an active community of autotrophic denitrifiers, the actual sulfide concentration would be negligible and the intermediate nitrite produced could be consumed by anammox.

In this study we investigated whether anammox activity could be linked to autotrophic denitrification. This hypothesis was tested under laboratory conditions by using a co-culture of anammox bacteria and the known autotrophic denitrifier *Sulfurimonas denitrificans* DSM 1251 (Timmer-Ten Hoor, 1975) that was supplied with only nitrate and ammonium, but not nitrite under a continuous influx of sodium sulfide. We hypothesized that denitrification would convert sulfide and nitrate, thereby allowing anammox to stay active despite an influx of sulfide and supply them with substrate (NO_2^-) under conditions of partial denitrification.

Results and Discussion

Sulfide toxicity in anammox bacteria

The sensitivity of anammox bacteria enriched from wastewater treatment plants towards short time hydrogen sulfide exposure has been described in the literature several times concluding that the half maximal inhibitory concentration (IC50) is in the low mM range (van de Graaf *et al.*, 1995; Dapena-Mora *et al.*, 2007; Jin *et al.*, 2013). In the environment these critical inhibitory concentrations seem to be even lower with low µM concentrations completely inhibiting anammox activity in sediments (Jensen *et al.*, 2008). This would also explain the absence of anammox from sulfidic waters as has been reported in several studies (Dalsgaard *et al.*, 2005; Lam *et al.*, 2007).

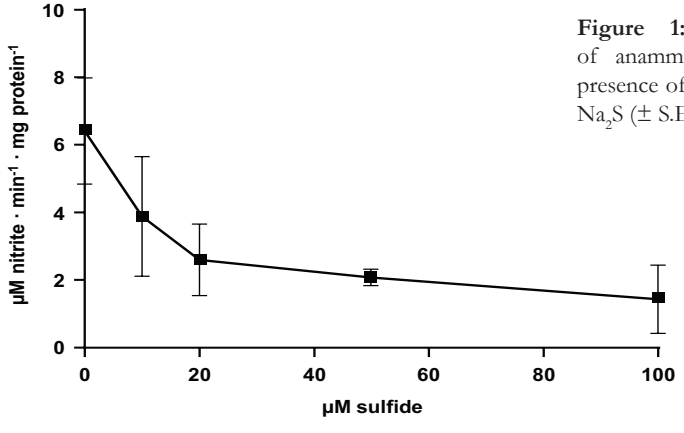

Figure 1: Nitrite-reducing activity of anammox planktonic cells in the presence of different concentrations of Na_2S (± S.E. of 4 replicates).

We tested the nitrite-reducing capacity of the free-living planktonic anammox cells in the presence of increasing sulfide concentrations to determine the IC50 value of the culture we later on used to inoculate the reactor system. Short-term exposure (2 h) had a strong effect on anammox activity (Fig. 1) resulting in an IC50 as low as 10 (± 4) µM. This is significantly lower than previously described inhibitory concentrations of enrichment cultures.

Several reasons might explain these results: Previous experiments were done with aggregated biomass from wastewater treatment plants. It is known that biofilm formation allows for a much higher resistance to toxic agents which might explain the higher maximal inhibitory concentration of granules compared to planktonic cells (Costerton *et al.*, 1987). Although the exact molecular mechanism of sulfide toxicity is not resolved yet, it has been shown that H_2S reversibly inhibits cytochromes and other heme-containing proteins (reviewed in Kabil and Banerjee, 2010). Thus the high sensitivity of anammox to sulfide is probably due to fact that anammox bacteria harbor an exceptionally high number of heme-containing proteins Strous *et al.*, 2006; Kartal 2011).

Although it has been observed that the inhibitory effect of H_2S on other heme-containing proteins such as the mitochondrial cytochrome oxidase is reversible, (Petersen, 1977; Cooper and Brown, 2008) no increase in nitrite-reducing activity could be observed when we continued the experiment for up to 6 h. This is despite the fact that sulfide could no longer be detected in incubations with the lowest original sulfide concentrations. Additionally, we performed a continuous culture experiment to study the long-term effects of sulfide exposure on our anammox culture. Sulfide was initially added at a loading rate of ~70 $\mu M \cdot d^{-1}$ and instantly resulted in a drop in anammox activity which was evident from the accumulation of nitrite. Further, a significant drop in optical density (OD_{600}) from 0.32 to 0.18 was observed within 7 days, which pointed to cell lysis (data not shown).

Co-cultivation of anammox and sulfide oxidizers

Due to the fact that our anammox cultures were very sensitive to low sulfide concentrations, instead of stimulating the growth of sulfide oxidizers that could be present in the anammox culture, we decided to start a co-culture of *Sulfurimonas denitrificans* DSM 1251 (Timmer-Ten Hoor, 1975) and anammox bacteria by introducing the anammox bacteria to a stable culture of sulfur oxidizers. *S. denitrificans* was precultured anaerobically in an adapted anammox medium using sulfide and nitrate as substrates. The initial addition of sulfide (70 $\mu M \cdot d^{-1}$) lead to the visible formation of elemental sulfur. The supply of excess nitrate concentrations caused a transient nitrite accumulation (10-13 μM) as a result of incomplete denitrification (Fig. 2).

When the culture was stably consuming all supplied sulfide (effluent [HS^-] <2μM) anammox biomass was added at the ratio of one part anammox and three parts *S. denitrificans* according to OD_{600} values (day 0). As an immediate result, the nitrite concentration in the effluent decreased below

the detection limit (5 μM). At day 27, the influent sulfide load was increased (267 $\mu M \cdot d^{-1}$) leading to an increase in the consumption of ammonium and nitrate, while sulfide was still below the detection limit. When nitrate became limiting (day 40), low concentrations of nitrite could be measured (~ 5 μM), possibly caused by the inhibition of anammox activity by residual sulfide (not determined) that accumulated because nitrate concentrations were insufficient to consume it all. To facilitate a stable operation, the reactor was run under sulfide limitation by adding excess nitrate. During this phase the reactor consumed 1.27 mol NO_3^- and 0.83 mol NH_4^+ per mol of HS^-.

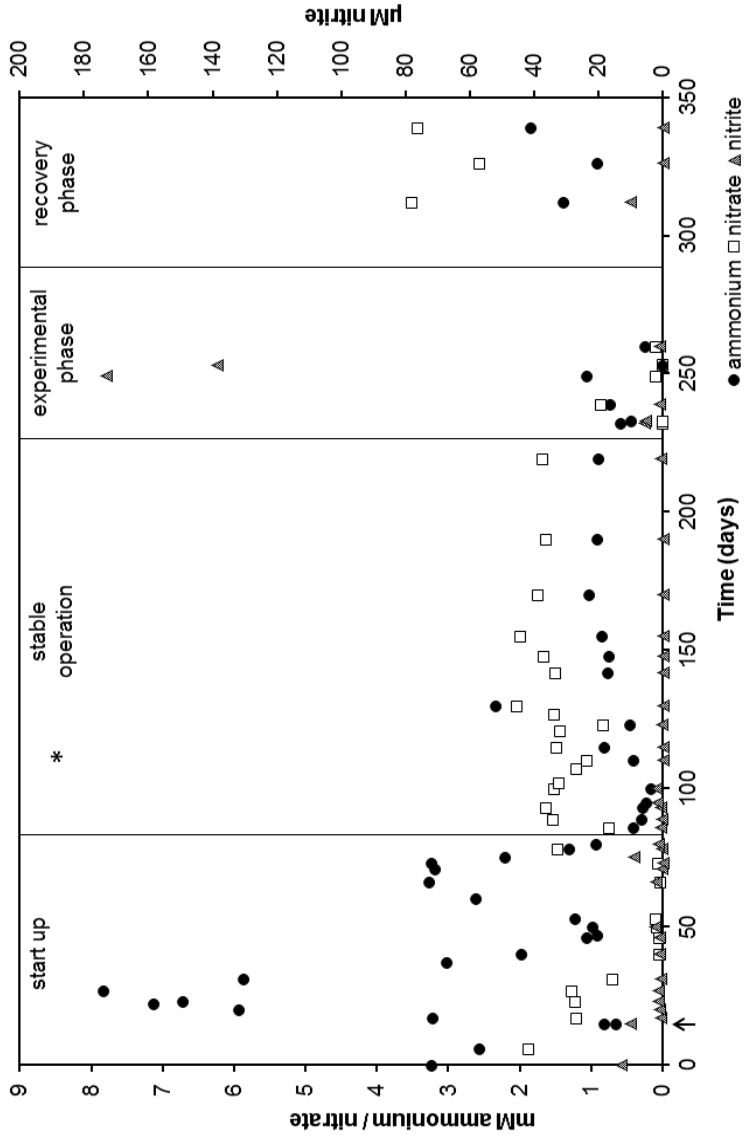

Figure 2: Effluent concentrations of ammonium, nitrate and nitrite of the reactor system hosting the co-culture during different stages in the experiment. The arrow (↑) indicates the day of anammox addition, asterisk (*) the day of sampling for metagenomic analysis. Sporadic sulfide measurements indicated concentrations below the detection limit.

Based on the assumption that anammox bacteria were responsible for all ammonium conversion, anammox bacteria were estimated to contribute 66 ± 2% to the nitrogen loss from the reactor system. Afterwards the reactor was alternatively limited in nitrate or ammonium (experimental phase) to allow quantification of the activity of the two guilds of microorganisms and their relative contribution to the nitrogen fluxes with stable isotopes. Short term nitrate limitation lead to a minor nitrite accumulation (~ 6 μM) that was consumed as soon as the nitrate influent was restored. Periods of combined nitrate and ammonium starvation (day 253) caused sulfide (not determined colorimetrically) and nitrite accumulation (~180 μM). When the feed was adapted to the stable operation phase, the reactor gradually recovered.

Assessing the community by shotgun metagenomic sequencing, FISH and PCR

The initial composition of anammox biomass in the reactor consisted for about 50% of *Kuenenia stuttgartiensis* and 50% *Scalindua profunda* which had been added to the running reactor of autotrophic denitrifiers in a ratio of 1:3. To confirm the presence of *Sulfurimonas dentrificans* and both anammox genera as the dominant players in the reactor, we sequenced the metagenome of the whole reactor during the stable operation phase (at day 133). Surprisingly, only 3502 of 3.847.959 reads (0.09%) could be mapped to *Sulfurimonas denitrificans*, indicating that this autotrophic denitrifier was lost from the reactor even though the reactor was still oxidizing sulfide. Further analysis of the metagenome showed that a Gammaproteobacterium was dominant in the reactor, which possibly took over the role of *S. denitrificans*. 43.9% of all reads could be mapped to the genome of *Sedimenticola selenatireducens* DSM 17993 (Narasingarao and Häggblom, 2006) with a similarity fraction of 0.9. This bacterium was originally isolated as a selenate reducer, but was also able of anaerobic respiration of acetate and pyruvate with nitrate or nitrite. Using the 16S rRNA gene consensus sequence of *Sedimenticola* sp., specific probes and primers were designed to target these organisms and determine when the shift from *Sulfurimonas denitrificans* to *Sedimenticola* sp. had taken place. The initial sample taken for FISH analysis after addition of the anammox biomass (day 0) could not conclusively show the presence of *Sedimenticola* sp. (Fig. 3A). Nevertheless, these microorganisms established themselves quickly and were the most abundant bacteria within the reactor system within 133 days (Fig. 3C). *Sedimenticola* sp. remained the dominant sulfide oxidizer throughout the course of the experiment accounting for more than 70% of the bacterial biomass (Fig. 3 C&D). To trace the source of these *Sedimenticola* sp. and their diversity, DNA was isolated from the original DSMZ biomass, the reactor

biomass from just before anammox addition, the anammox inoculum and the co-culture after 133 days. *Sedimenticola* sp. was detected in all samples and all sequenced clones had a similarity of >98% with *Sedimenticola selenatireducens* on 16S rRNA gene level. The DSMZ original culture as well as our anammox inoculum did also contain *Sedimenticola*-like sequences.

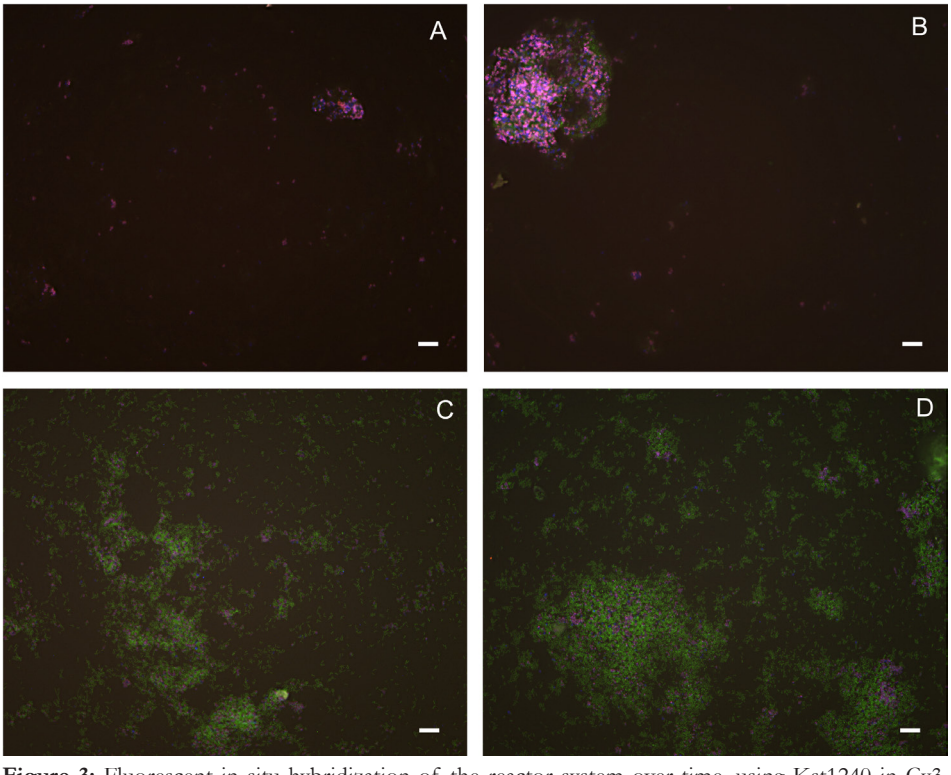

Figure 3: Fluorescent in situ hybridization of the reactor system over time, using Kst1240 in Cy3, AMX820 in Cy5 and Gam781 in FLUOS. *K. stuttgartiensis* is visible in pink due to double binding of Kst1240 and AMX820, other anammox bacteria color blue and *Sedimenticola* sp. is visible in green. A shows the day of anammox addition (day 0), B, C and D show the community after 25, 133 and 204 days respectively. The scale bar represents 5 μM.

To determine whether the *Sedimenticola* sp. in our reactor system had the genetic potential to perform autotrophic denitrification, which had not been described for *Sedimenticola selenatireducens*, we assembled all mapped reads into 580 contigs and analyzed the assembled genome for key genes of sulfur oxidation and denitrification. The *Sedimenticola* sp. present in the reactor harbored a range of genes involved in the oxidation of reduced, inorganic sulfur compounds, which could facilitate the complete oxidation of sulfide to sulfate (Supplementary Table 2). Sulfide can be converted to elemental sulfur (S^0) by sulfide dehydrogenase (*fccAB*). The dissimilatory sulfite reductase (*dsr*)

complex allows the conversion of S^0 to sulfite, which can in turn be oxidized directly to sulfate by a sulfite oxidase or in a two-step process including adenosine 5'-phosphosulfate reductase (*apr*) oxidizing sulfite to adenosine 5'-phosphosulfate (APS) and adenosine 5'-triphosphate sulfurylase (*sat*) oxidizing APS to sulfate. Several other sulfur-oxidizing bacteria also harbor both sulfite-oxidizing pathways granting them a very adaptable dissimilatory sulfur metabolism (Kappler and Dahl, 2001). The genome of the *Sedimenticola* strain also encoded the Sox pathway (*soxABXYZ*) for the oxidation of thiosulfate which might be an important intermediate in areas with a low sulfide to oxygen ratio or in sediments due to the oxidation of free and iron-bound sulfides (Chen and Morris, 1972; Moses *et al.*, 1987; Schippers and Sand, 1999). Furthermore, genes encoding for an almost complete denitrification pathway [a membrane-bound (*nar*) and a periplasmic (*nap*) nitrate reductase, a cytochrome cd_1 nitrite reductase (*nirS*) and nitrous oxide reductase (*nosZ*)] were also detected in the genome assembly of *Sedimenticola* sp. (Supplementary Table 2). The catalytic subunit of the nitric oxide reductase (*norB*) could not be retrieved from the metagenome, but several nitric oxide reductase activation proteins (*norDEQ*) were found. That and the fact that *S. selenatireducens* DSM 17993 seems to encode even 2 copies of *norB* lead to the assumption that the gene must also be present in our strain. For almost all key functional genes, the similarity with the type strain on nucleotide level was more than 95% (Supplementary Table 2).

The exact reasons causing the disappearance of *Sulfurimonas denitrificans* remain elusive, but there seems to be a physiological difference between gamma- and epsilonproteobacterial sulfur oxidizers. *Sulfurimonas denitrificans* has been described to perform the complete reduction of nitrate to dinitrogen gas (Timmer-Ten Hoor, 1975) and also its close relative *Sulfurimonas gotlandica*, which plays an important role at the redoxcline in the Baltic Sea did accumulate neither nitrite nor zero-valent sulfur and could grow on sulfide only if the concentration was below 10 µM (Bruckner *et al.*, 2012; Grote *et al.*, 2012). The *Sedimenticola* strain could therefore have outcompeted *Sulfurimonas denitrificans* by using sulfide as electron donor. Also the ability to conserve enough energy from partial denitrification might have been of importance upon the addition of anammox bacteria, as anammox bacteria have a very high affinity for nitrite and might outcompete a complete denitrifier (Strous *et al.*, 1999, Kartal *et al*, 2007; Kartal *et al*, 2008).

Metagenome analysis and FISH also pointed to a shift within the anammox population. Approximately 10% of all reads could be mapped to anammox genomes (similarity fraction 0.85) although FISH revealed that actual

anammox numbers were much higher (>25%) (Fig. 3A-D). According to the mapping results, the anammox population in the bioreactor consisted for about 27% of *Scalindua profunda* and about 73% of *Kuenenia stuttgartiensis*. The simultaneous use of a *K. stuttgartiensis*-specific probe (Kst1240) and the general anammox probe (AMX820) in FISH revealed similar results, showing a clear dominance of *K. stuttgartiensis* after 133 days despite the marine settings of the reactor system (Fig. 3C). Although we used an artificial medium for the enrichment of marine anammox bacteria, salt and nitrite limitation did obviously not select against *K. stuttgartiensis*. It is known that freshwater anammox bacteria grow in salt concentrations up to 30 $g \cdot l^{-1}$ (Kartal *et al.*, 2006; Liu *et al.*, 2009), but still the genus *Scalindua* sp. seems to dominate natural marine environments (Penton *et al.*, 2006; Schmid *et al.*, 2007; Woebken *et al.*, 2008) raising the question what the determining selective factor is between freshwater and marine anammox genera.

Contribution to N_2 production in the bioreactor

The interaction between autotrophic denitrification and anammox and the relative contribution of these processes to the nitrogen loss from the bioreactor was monitored with differential addition of stable isotopes. All experiments were performed with sulfide, NO_3^- and NH_4^+. When $^{15}NH_4^+$ and $^{14}NO_3^-$ were used as substrates, anammox activity would result in the production of $^{29}N_2$, whereas denitrification would lead to the production of $^{28}N_2$. On the other hand, when $^{14}NH_4^+$ and $^{15}NO_3^-$ were used as substrates, denitrification and anammox would produce $^{30}N_2$ and $^{29}N_2$, respectively.

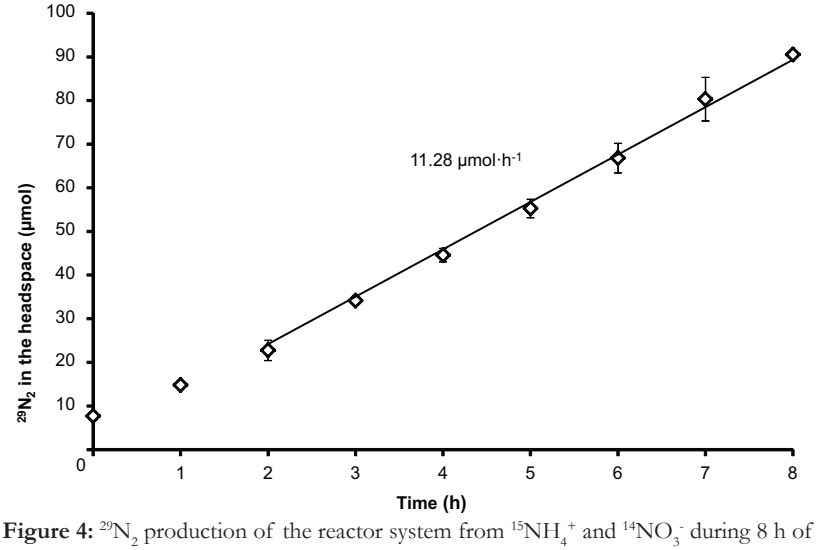

Figure 4: $^{29}N_2$ production of the reactor system from $^{15}NH_4^+$ and $^{14}NO_3^-$ during 8 h of operation as a fed batch (headspace volume = 1.5 l).

Upon the addition of sulfide, $^{15}NH_4^+$ and $^{14}NO_3^-$ to the reactor, $^{29}N_2$ was produced with a rate of 11.3 ± 1.3 μmol·h^{-1} (Fig. 4). When $^{14}NH_4^+$ and $^{15}NO_3^-$ were supplied, both processes were active simultaneously as $^{29}N_2$ and $^{30}N_2$ concentrations increased with rates of 6.4 μmol·h^{-1} and 3.3 μmol·h^{-1}, respectively (Fig. 5).

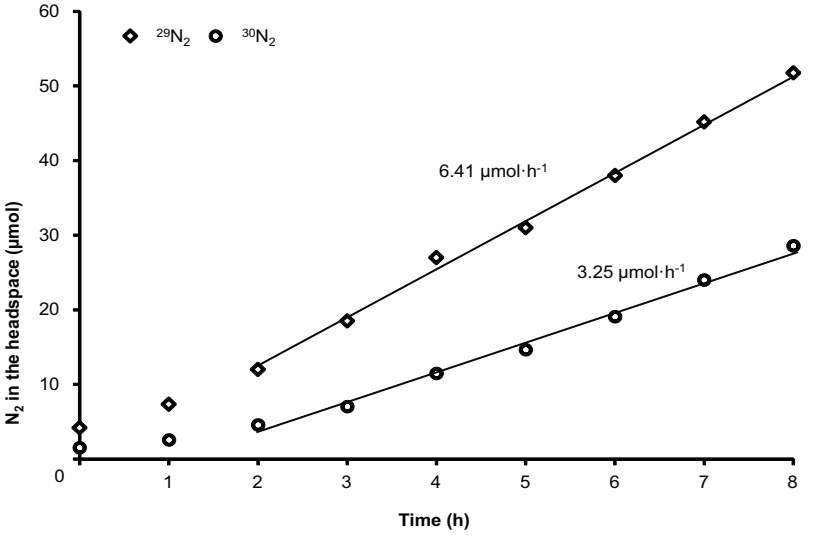

Figure 5: $^{29}N_2$ and $^{30}N_2$ production of the reactor system from $^{14}NH_4^+$ and $^{15}NO_3^-$ during 8 h of operation as a fed batch (headspace volume = 1.5 l).

The production of NO and N_2O as intermediates was not detected at any time. Anammox activity was lower when the labeling experiment was conducted with $^{15}NO_3^-$. This might be due to two factors: The initial starvation of the reactor for nitrate before the addition of $^{15}NO_3^-$ resulted in sulfide accumulation, which has a negative effect on anammox activity therefore rendering their activity slightly lower. Further, ammonium starvation before the addition of $^{15}NH_4^+$ generally resulted in residual nitrate in the reactor. As there was a higher demand for nitrate than for ammonium, because both processes require it as a substrate, the actual nitrate availability was higher when labeled ammonium was added, which could lead to a higher anammox activity.

Batch activity assays

To test the activity of the co-culture under more controlled conditions batch activity assays were performed with differential labeling ammonium and nitrate and in the presence and absence of sulfide as electron donor. To avoid disturbance of the signal by heterotrophic denitrification that could

use nitrate with degradation products as electron donors, the duration of the experiment was limited to 24 h. A total of 150 μM sulfide was added in intervals of at least 2 h during this period.

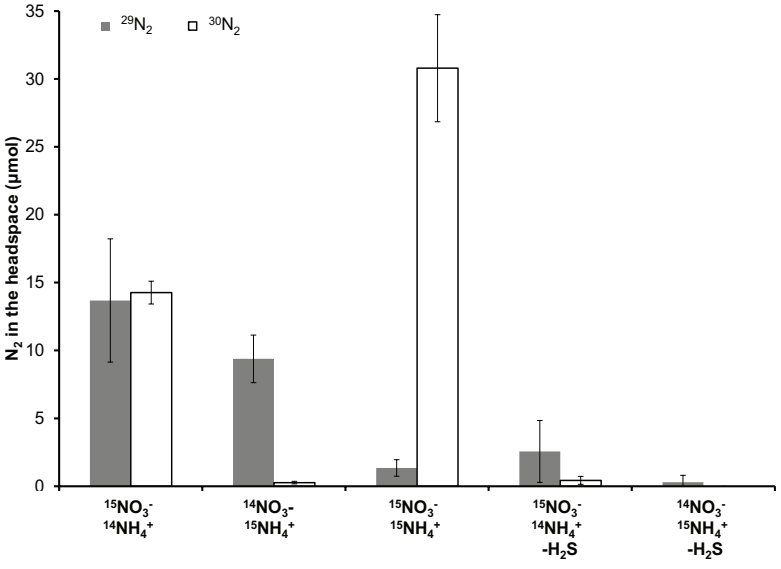

Figure 6: $^{29}N_2$ and $^{30}N_2$ production in batch activity assays using co-culture biomass and differentially labeled substrates.

The interaction between anammox and autotrophic denitrification was strongly dependent on sulfide as there was no net production of dinitrogen gas in the absence of sulfide (Fig. 6). We performed five biological replicate incubations. One of which consumed up to 150 μM nitrate explaining the relatively high standard error with $^{15}NO_3^-$ in the absence of sulfide, but there was a significantly lower nitrate consumption and N_2 production without sulfide. This showed that anammox bacteria depended on the conversion of nitrate to nitrite by autotrophic denitrification and under these conditions did not directly metabolize nitrate to produce NO or dinitrogen gas themselves. When sulfide was added, the culture consumed 210 ± 15 μM nitrate and transiently produced 25-30 μM of nitrite during the first 24 h (data not shown). In the absence of sulfide the nitrite produced was much lower (<10 μM). A slightly lower anammox activity was observed in incubations with $^{15}NH_4^+$ compared to those with $^{15}NO_3^-$ (Fig. 6). We assume that this might be the result of an overestimation of anammox activity in case of $^{15}NO_3^-$-labeling, as there might be a small pool of unlabeled nitrate present. This unlabeled nitrate might be stored internally and could be used to as an electron acceptor for denitrification thereby contributing to the $^{29}N_2$ pool. The increase of $^{29}N_2$

also in the absence of sulfide and the production of small amounts of $^{29}N_2$ when both substrates were labeled also indicated a possible internal nitrate storage (Fig. 6). In both reactor and batch labeling experiments, contribution of anammox to the nitrogen loss was in the same range (65-75%).

Environmental significance of partial autotrophic denitrification coupled to anammox

In marine environments an interaction between denitrification and anammox was previously shown in continental shelf sediments where $^{29}N_2$ gas was produced in the presence of $^{15}NO_3^-$, indicating that nitrate was first converted to $^{15}NO_2^-$ before it was combined with ammonium to form $^{29}N_2$ (Dalsgaard *et al.*, 2003). Additional evidence on the possible importance of this process in marine environments originated from a metagenomics and -transcriptomics study in the OMZ off of the coast of Chile: Anammox and gammaproteobacterial sulfur-oxidizers of the so-called SUP05 subgroup seemed to be present in the same layer of the water column (Canfield *et al.*, 2010; Stewart *et al.*, 2012). Although it is generally stated that anammox is absent from sulfidic waters (Dalsgaard *et al.*, 2005; Lam *et al.*, 2007), recent studies describe the presence of certain phylotypes of the marine *Scalindua* sp. in the lower suboxic zone of the Black Sea where sulfide concentrations were up to 10 µM (Wakeham *et al.* 2012, Fuchsmann *et al.* 2012, Kirkpatrick *et al.*, 2012,). This could mean that there was a certain divergence in the adaptation towards sulfide. A very adaptive metabolism responding quickly to a changing environment might be one of the attributes determining the success of *Scalindua* phylotypes in marine habitats with fluctuating conditions. An example of such an anammox activity was described in the Baltic Sea: although anammox activity could not be shown in the presence of measurable sulfide, these microorganisms contributed significantly to the total N_2 production after a major inflow event of oxygenated North Sea water that resulted in a sulfide free zone (Hanning *et al.*, 2007). Anammox bacteria might enter dormancy in times of unfavorable conditions, but regain their activity quickly as soon as conditions improve (Jones and Lennon, 2010). Alternatively, they might switch to a different metabolism (Strous *et al.*, 2006, Kartal *et al.*, 2007, 2008).

In marine sediments the sulfur-fueled nitrogen cycle is an important process (Schulz *et al.*, 1999, Campbell *et al.*, 2006). In the deep sea sediments of the Guaymas Basin, anammox bacteria were detected within sulfidic sediments (Russ *et al.,* 2013). Their numbers were highest at the sediment interface where sulfide concentrations were lowest, but still functional genes of sulfide oxidizers could be retrieved from the same depth, again showing that these

two organisms could potentially share the same niche. Also in freshwater ecosystems anammox and autotrophic denitrifiers occur in the same water layer. Although direct exchange of intermediates has not been determined, both processes were actively taking place (Wenk *et al.*, 2013).
From the applied point of view, such a coupling might offer interesting opportunities for the treatment of ammonium-rich, sulfidic wastewater. As sulfide leads to partial inhibition of NO reduction and a strong inhibition of N_2O reduction in denitrifying cells, a scenario with a significantly lower release of NO and the green gas N_2O from such systems would be possible (Sørensen *et al.*, 1980, Schönharting *et al.*, 1998, Dalsgaard *et al.*, 2013). In our experiments we never saw the release of any other gas than N_2, because anammox bacteria have a very high affinity for nitrite so most of it has been consumed by anammox before it can be transformed into NO or N_2O by denitrification (Strous *et al.* 1999). The system would however be sensitive to high sulfide loads or ammonium limitation.
The complex interactions of the nitrogen cycle bacteria is an interesting example of how complex the determination of fluxes can be, with many different players and transformations to consider. Using a laboratory scale model system we showed that the anammox bacteria could indeed be fueled by nitrate reduction at the constant influx of sulfide and ammonium. We believe that this interaction between anammox and denitrification (autotrophic and also heterotrophic) could play an important role in the environment, especially in zones of cryptic sulfur cycling where sulfide is at sub μM level.

Experimental procedures

Fluorescence In Situ Hybridization
Biomass was fixed in a 4% w/v paraformaldehyde solution for 1-3h on ice. After washing cells were stored at -20 ºC in a 50% v/v 1 X PBS and 50% v/v ethanol solution. Probe sequences and optimal formamide concentrations for hybridization are listed in Supplementary Table 1. Staining with DAPI was included as a positive control.

DNA isolation and 16S PCR
Genomic DNA was isolated from different samples using the PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Inc.) according to the manufacturer's protocol.
All PCR amplifications were performed in a total volume of 25 μl using 12.5 μl PerfeCTa® SYBR® Green FastMix (Quanta), 0.4 μM forward/reverse primer (Sed784F-5'-GCCAAGATGCTCCCAACAAC and Sed1249R-5'-

AGCCTGATCCAGCAATACCG) 2 µl of template and 10.5 µl of DEPC-treated H_2O. Amplification was initiated with a denaturation step at 94 °C for 2 min and continued with a standard amplification program of 35 cycles (30 sec 94 °C; 40 sec 55 °C; 1 min 72 °C). The final elongation step was done at 72 °C for 10 min.

Next generation sequencing

DNA was extracted from 24 ml co-culture biomass using the PowerSoil® DNA isolation kit (MO BIO, USA) following the protocol. DNA quality was checked by agarose gel electrophoresis (1%) with ethidium bromide. Concentrations were determined spectrometrically using the NanoDrop (Thermo Scientific). 1 µg genomic DNA was sheared for 7 minutes using the Ion Xpress™ Plus Fragment Library Kit (Life technologies, USA) following the manufacturer's instructions. Further library preparation was performed using the Ion Plus Fragment Library Kit (Life technologies, USA) following manufacturer's instructions. Size selection of the library was performed using an E-gel 2% agarose gel (Life technologies, USA) resulting in a mean fragment length of 339 bp. Emulsion PCR was done using the Onetouch 200bp kit and sequencing was performed on an IonTorrent PGM using the Ion PGM 200bp sequencing kit and an Ion 318 chip (Life technologies, USA), resulting in 4.349.930 reads of average length 167 bp.

Analysis of reads was performed using CLC genomics workbench 6.0.2 (CLCbio, Denmark). The reads were length and quality score trimmed (>50bp, 0.05) resulting in a dataset of 3.847.959 reads. 43.8% of all reads could be mapped to the genome of *Sedimenticola selenatireducens* DSM 17993 with mismatch penalty 2, In/Del penalties 3 and 90% identity over 50% of the read length. The subset was extracted and a de novo assembly included in the program mapping the reads back to the contigs (Length fraction 0.5, similarity fraction 0.8) was performed using the default settings. This resulted in 580 contigs. These contigs were submitted to the RAST sever for annotation (Aziz *et al.* 2008).

Batch activity assays for IC50 determination

Active anammox biomass (50% *Scalindua profunda*, 50% *Kuenenia stuttgartiensis)* planktonic cells were harvested from a membrane reactor. 10 ml cell biomass were added to a 30 ml serum bottle and a total concentration of 2 mM ammonium chloride and 2 mM sodium nitrite were added as substrate. Sodium sulfide was added in concentrations of 5, 10, 20, 50 and 100 µM from a 100 mM anaerobic stock solution. The bottles were sealed with 5mm butyl

rubber stoppers and made anoxic by alternatively applying underpressure and flushing with Argon several times, ultimately maintaining an overpressure of 1 bar on the bottles. Incubation was done at room temperature and shaken at 200 rpm for 2 h, during which the decrease in nitrite and ammonium was followed.

Analytical methods

Nitrite was measured colorimetically at 540 nm after a 15 min reaction of 1 ml sample (0.1 – 0.5 mM nitrite) with 1 ml 1% sulfanilic acid in 1 M HCl and 1 ml 0.1% naphtylethylene diaminedihydrochloride. Ammonium was measured at 420 nm on a Cary Eclipse Fluorescence Spectrophotometer after reaction with 10% ortho-phthaldialdehyde as has been described previously (Taylor *et al.*, 1974). Sulfide concentrations were determined by detection of hydrogen sulfide as methylene blue at 460 nm (Fonselius *et al.*, 2007). Nitrate was measured by injection of 10 µl sample into an HP Agilent 1050 series auto sampler using a sodium hydroxide solution at a flow of 1.5 $ml \cdot min^{-1}$ as the liquid phase. The anions were eluted via an isocratic method with 30 mM NaOH in 6 min and separated on a 4x250mm Ionpac AS11-HC (Dionex, UK) column. The UV/Vis absorbance spectrum of nitrate was measured at 220 nm.

Reactor operation

A fermentor (working volume 1.5 l) was used to gradually adapt *Sulfurimonas denitrificans* DSM 1251 to higher salt concentrations (2.5%) and sulfide as electron donor. Medium generally used to cultivate marine anammox bacteria (van de Vossenberg *et al.*, 2008) was adjusted to a salt content of 37.5 $g \cdot l^{-1}$ and added to the reactor with a flow of 150-200 $ml \cdot d^{-1}$. The pH was controlled at 7.1 with a sterile 100 $g \cdot l^{-1}$ $KHCO_3$ solution. Sodium sulfide was added separately from an anaerobic 6 mM stock with a flow of 90-100 $ml \cdot d^{-1}$. The reactor was operated under anaerobic conditions by constant flushing with Arg/CO_2 (10 $ml \cdot min^{-1}$). Sulfide was limiting with influent nitrate and ammonium concentrations of 8 mM NO_3^- and 5 mM NH_4^+ during stable operation. During the limitation experiments NO_3^- or NH_4^+ concentrations were lowered until either NO_3^- or NH_4^+ was below the detection limit. Afterwards influent nitrate and ammonium concentrations were either 2 mM $^{15}NO_3^-$ and 4 mM $^{14}NH_4^+$ under nitrate-limiting conditions or 4 mM $^{14}NO_3^-$ and 2 mM $^{15}NH_4^+$ under nitrate-limiting conditions, corresponding to a total of ~ 125 µM labelled substrate to the reactor system in 8 h. The reactor was sealed and the Arg/CO_2 was switched off. Gas samples were taken from a

sampling port every hour.

Activity assays

For batch activity assays biomass was harvest from the co-culture by centrifugation at 4000 rpm (10 min, 4 ºC) and washed once before resuspending it in fresh medium (pH 7.1) supplemented with 10 mM $KHCO_3$. Total concentrations of 1 mM $^{14}NO_3^-$ or $^{15}NO_3^-$ and $^{14}NH_4^+$ or $^{15}NH_4^+$ were added. Anaerobic conditions were obtained by alternately applying vacuum and flushing with Arg/CO_2. An overpressure of 1 bar was maintained. Sodium sulfide was added in portions of 20 μM (to avoid a toxicity effect) to a total concentration of 150 μM. Incubations were shaken with 150 rpm at 20 ºC.

For whole reactor activity assays the reactor was starved in either nitrate of ammonium before feeding either 2 mM $^{15}NO_3^-$ and $^{14}NH_4$ or $^{14}NO_3^-$ and $^{15}NH_4^+$ via the influent at the rates described above. The avoid washout of labeled gas compounds the reactor was operated as batch during 8 h of measurement. The production of $^{28}N_2$, $^{29}N_2$ and $^{30}N_2$ was followed by gas chromatography coupled to mass spectrometry on an Agilent 6890/5975c MSD. For the quantification of N_2 standard curves a commercially available gas mixtures was used containing 1% N_2. Nitrate, ammonium and nitrite were determined as described earlier.

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Supplementary Table 1: Description of FISH probes used in this study.

Name	Target organism	Sequence (5'- 3')	Labeling dye	Formamide	Reference
Kst1240	*Kuenenia stuttgartiensis*	CTTAGCATCCCTTTGTACCGACC	Cy3	20-30%	This study
AMX820	All anammox	AAAACCCCTCTACTTAGTGCCC	Cy5	20-30%	Schmid et al. 20(
Gam781	*Sedimenticola*-like	AGAGCCAAGATGCTCCCAAC	FLUOS	20-40%	This study
Gam784	*Sedimenticola*-like	GCCAAGATGCTCCCAACAAC	FLUOS	20-40%	This study

Supplementary Table 2. Genes of *Sedimenticola* sp. involved in nitrate reduction and sulfide oxidation

Pathway	Gene number	Contig	Gene	% *S. selenatireducens* (blastn)	Function
Cytoplasmic dissimilatory nitrate reduction	2415	396	*narG1*	91	Respiratory nitrate reductase alpha chain
	2985	54	*narG2*	98	
	2986	54	*narH*	97	Respiratory nitrate reductase beta chain
	2988	54	*narJ*	98	Respiratory nitrate reductase delta chain
	2989	54	*narI*	95	Respiratory nitrate reductase gamma chain
Periplasmic dissimilatory nitrate reduction	1643	278	*napA*	90	periplasmic nitrate reductase, large subunit
	2257	370	*napH1*	97	Polyferredoxin
	3272	69	*napH2*	96	
	2258	370	*napB*	94	Nitrate reductase cytochrome c550-type subunit
	2662	454	*napA*	97	Periplasmic nitrate reductase precursor
	2663	454	*napF*	94	Ferredoxin-type protein
	2259	370	*napC1*	97	Cytochrome c-type protein
	2882	515	*napC2*	100	
	470	149	*napC3*	96	
	3271	69	*napG*	98	Ferredoxin-type protein
Dissimilatory nitrite reduction to NO	469	149	*nirS*	97	cytochrome cd1 nitrite reductase
	473	149	*nirC*	96	cytochrome c55X precursor
	474	149	*nirF*	98	heme d1 biosynthesis protein
	475	149	*nirD*	98	heme d1 biosynthesis protein
	476	149	*nirL*	97	heme d1 biosynthesis protein
	477	149	*nirG*	97	heme d1 biosynthesis protein
	732	177	*nirE*	99	uroporphyrinogen-III methyltransferase
	2480	405	*nirJ1*	94	heme d1 biosynthesis protein
	2694	458	*nirJ2*	97	
	2481	405	*nirN1*	94	cytochrome cd1 nitrite reductase associated cytochrome
	2693	458	*nirN2*	97	
Nitric oxide reduction	390	14	*norE1*	96	nitric oxide activation protein
	552	158	*norE2*		
	554	158	*norQ*	98	nitric oxide activation protein
	556	158	*norD*	98	nitric oxide activation protein
Nitrous oxide reduction	84	69	*nosD*	99	nitrous oxide reductase maturation protein
	3263	69	*nosZ*	96	nitrous oxide reductase
	3270	69	*nosD*	98	nitrous oxide reductase maturation protein
	3276	69	*nosY*	97	nitrous oxide reductase maturation trans-membrane protein
Sulfide oxidation	2723	469	*sqr*	97	Sulfide-quinone reductase
	2388	390	*fccA1*	97	Cytochrome subunit of sulfide dehydrogenase precursor
	560	158	*fccA2*	98	
	2389	390	*fccB1*	98	Sulfide dehydrogenase flavoprotein chain
	561	158	*fccB2*	98	
Thiosulfate oxidation	3121	59	*soxB*	91	Sulfur oxidation protein
	3123	59	*soxZ1*	95	Sulfur oxidation protein
	1571	266	*soxZ2*	95	
	3124	59	*soxY1*	94	Sulfur oxidation protein
	1570	266	*soxY2*	93	
	3127	59	*soxA1*	95	sulfur oxidation protein
	1569	266	*soxA2*	94	
	3128	59	*soxX1*	97	Sulfur oxidation protein
	1568	266	*soxX2*	92	

Chapter 4

Comparative gene expression on a co-culture of anaerobic ammonium and sulfur-oxidizing bacteria under ammonium and nitrate limitation

Russ, L., van Alen, T., Jetten, M.S.M., Op den Camp, H.J.M. and Kartal, B.

Summary

Comparing the expression of genes under different growth conditions can provide valuable insight into the response of a microorganism to changing environmental conditions. To elucidate the effect of nitrate- and ammonium-limiting conditions on anammox bacteria and autotrophic denitrifiers we subjected a previously described co-culture of a sulfide-dependent denitrifier and anammox bacteria to differential limitations and compared the short-term changes in the transcriptome of the key players to those under standard conditions. Under ammonium-limiting conditions 762 genes of *Scalindua sp.* were either up- or downregulated representing about 15% of the total predicted genes. As ammonium was an essential substrate its low concentrations led to the downregulation of genes involved in energy conservation, transcription, translation, protein biosynthesis, cell division and carbon fixation. The ammonium transporting genes (*amtB*) were significantly upregulated (7-128-fold) under ammonium limitation. The two encoded *focA*-like formate/nitrite transporters were also upregulated (5-10-fold), replacing the *nirC*-like scal00416 as the most highly expressed nitrite transporter. Also genes involved in the formation of flagella were highly upregulated under ammonium limitation to ensure motility under unfavorable conditions. Nitrate limitation did not have the same effect as differential expression of only 228 genes (4.6%) could be observed. *Sedimenticola sp.* differentially expressed 742 genes under nitrate limitation, which represents 17% of all its genes. The most significant changes were the downregulation of genes making up the membrane-bound nitrate reductase complex, genes involved in sulfur metabolism as well as energy conservation and central metabolism. For *Sedimenticola sp.*, ammonium limitation resulted in the up- or downregulation of 331 genes, including the upregulation of ammonium transporter genes and genes encoding nitrogen assimilation enzymes. This study presents the first insights into the interaction of anammox bacteria and its denitrifying partner at the molecular level by studying gene expression changes in response to ammonium and nitrate.

Introduction

Release of fixed nitrogen from (marine) ecosystems proceeds via two different pathways: denitrification and anaerobic ammonium oxidation (anammox). Both processes require suitable electron donors and oxidized N (i.e. nitrate or nitrite) to ultimately produce dinitrogen gas (N_2). Denitrification starts with the reduction of nitrate (NO_3^-) to nitrite (NO_2^-) followed by a stepwise reduction to dinitrogen gas ($NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$) where any of the intermediate products can also be released as end product. Denitrifiers use either an organic (heterotrophic) or an inorganic (autotrophic) compound as electron donor for N reduction. In the anammox process NO_2^- is first reduced to NO, which is then combined with ammonium (NH_4^+) to form hydrazine (N_2H_4), which is further oxidized to form N_2 (Strous et al., 2006; Kartal et al., 2011). NO_2^-, the electron acceptor in the anammox reaction, occurs in nature at low concentrations, and can be produced either via (micro)aerobic oxidation of ammonium or nitrate reduction. Both processes have been shown to indirectly contribute to nitrogen release by supplying significant amounts of NO_2^- to drive the anammox process (Kuypers et al., 2005; Francis et al., 2007; Lam et al., 2007; Lam et al., 2009; Lam and Kuypers, 2011). This implies that bacteria able to perform the anaerobic oxidation of ammonium are strongly dependent on other nitrogen transformations for the supply of substrates. To get a better understanding about the coupling of the anammox process to other nitrogen transformations, laboratory scale model systems have been used to simplify the interactions between selected partners and monitor their physiological responses: It was shown that ammonium oxidizing archaea and bacteria could indeed provide nitrite for anammox bacteria under oxygen limitation (Yan et al., 2012), as could a denitrifier closely related to *Sedimenticola selenatireducens* by reducing nitrate to nitrite at a continuous influx of sulfide as electron donor (Russ et al., 2014). Using such model reactor systems not only allows the study of the physiology of anammox and an interaction partner, but it also harbors the potential to unravel the effect of limitations of substrates as they might occur in nature to elucidate the response of microorganisms on a gene expression level by using comparative transcriptomics.

We previously showed that anammox activity could be linked to autotrophic denitrification. This was tested under laboratory conditions by using a co-culture of anammox bacteria and the *Sedimenticola selenatrireducens*-like denitrifier that was supplied with only nitrate and ammonium (no nitrite) under a continuous influx of sodium sulfide. Denitrification could convert sulfide and nitrate, thereby allowing anammox to stay active despite an influx

of sulfide and supply them with substrate (NO_2^-) under conditions of partial denitrification. This would suggest that at all times sulfide was the limiting substrate for denitrification as was nitrite for anammox. As natural, aquatic ecosystems can be dynamic and the microbial population has to adjust to a quickly changing environment it was chosen to limit the existing culture also in ammonium or nitrate in short term experiments (1-2 days) to monitor the first response in gene expression of anammox bacteria and their denitrifying partner to those limitations. Here we determined the changes in gene expression of anammox bacteria and *Sedimenticola sp.* in the model system that has been described previously (Russ et al., 2014) by comparing a control condition to NH_4^+- and NO_3^--limitation. Comparing the expression of genes under different growth conditions can give valuable insight into the response of a microorganism to physicochemical changes in the environment.

Results and Discussion

In the course of our experiments the culture was continuously under sulfide limitation. For this study, at different time points, the reactor was also limited in nitrate or ammonium. The reactor was sampled for transcriptome analysis after 1-2 days of being exposed to the limitation. Transcripts derived from the limitation conditions were compared to a control condition in which all added substrates were available in excess. The limitation experiments were repeated twice to achieve a biological replicate (hereafter called set 1 and 2).

Reactor operation

When the reactor was operated under the control conditions in our first experiment the influent contained 9 mM of nitrate and 7 mM of ammonium corresponding to a load of 240 μM $NO_3^- \cdot d^{-1}$ and 187 μM $NH_4^+ \cdot d^{-1}$. Sodium sulfide was added separately from an anaerobic 6 mM stock at a flow of 90-100 $ml \cdot d^{-1}$. The concentrations of ammonium and nitrate were adapted depending on the limitation and sulfide was limiting throughout the experiment. Under the control conditions the reactor had 5.1 mM nitrate, 30 μM ammonium (Fig.1A); NO_2^- and sulfide were below detection limit (<5 μM). Under ammonium-limiting conditions ammonium and sulfide concentrations in the reactor were below detection limit (10 and 5 μM, respectively), there was excess nitrate (3.7 mM) and nitrite accumulated in the reactor (191 μM). Nitrite accumulation under these conditions was due to the fact that the anammox bacteria were limited in ammonium and furthermore it suggested that under electron donor limitation (in this case sulfide), *Sedimenticola* sp. favored nitrate reduction to nitrite over complete denitrification. Under

nitrate-limiting conditions nitrite and nitrate were below detection limit (200 nM), there was a residual ammonium concentration of 25µM.

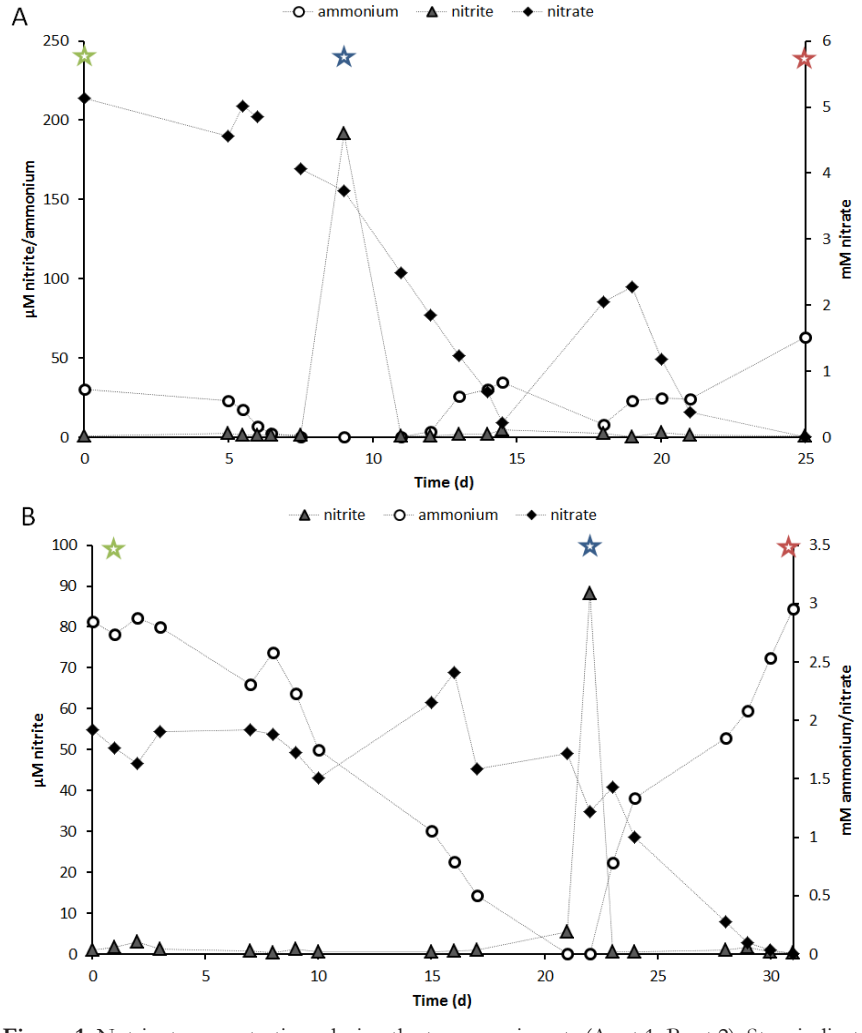

Figure 1: Nutrient concentrations during the two experiments (A set 1; B set 2). Stars indicate sampling points for control (green), ammonium-limited (blue) and nitrate-limited (red) conditions.

After the first sets of experiments were completed, the reactor was returned to the control conditions where both nitrate and ammonium were in excess and after a stabilization period, nitrate and ammonium limiting conditions were recreated separately to have biological replicates of the samples. In short, under control conditions, nitrite and sulfide were below detection limit and there was 1.7 mM nitrate and 2.7 mM ammonium in the reactor. When the reactor was ammonium limited, the ammonium concentration was

below detection limit (<10 μM), there was 1.2 mM nitrate in the reactor and nitrite accumulated to 88 μM. Under nitrate limitation nitrate, the nitrite and sulfide concentrations were below detection limit whereas the ammonium concentration in the reactor was 2.95 mM.

Validation of reproducibility

Monitoring transcriptome changes under different conditions require a robust and critical analysis pipeline to prevent misinterpretation of data. To this end we used two biological replicates as well as stringent conditions during data analyses. Mapping the datasets against the genomes of the key organisms *Sedimenticola sp.* and *Scalindua sp.* after trimming and removal of ribosomal RNA reads yielded an average of approximately 9% of the reads that could be mapped to *Sedimenticola sp.* of the first dataset and 12% of the second, whereas 14% and 5% respectively could be mapped to the *Scalindua sp.* reference genome. These rather low numbers might be due to the diverse population and the presence of tRNA reads in the dataset. After RNA-Seq analyses the RPKM values of all expressed genes from dataset 1 and 2 were plotted against each other to show the agreement of the biological replicates (Figs. 3A, B). The datasets of all expressed genes from the two biological replicates had a Spearman's correlation coefficient $r_s \geq 0.89$ pointing to the overall good reproducibility of the experiment.

Changes in the transcriptome of Sedimenticola sp. and Scalindua. sp. under ammonium and nitrate limitation

The molecular responses of the two key players in the continuous culture system to differential short-term substrate limitation was examined provided that a minimum of 20 reads mapped to a gene of interest in at least one of the conditions including the biological replicates and that the differential expression changed by at least 3-fold between the control condition and either nitrate or ammonium limitation. The changes in gene expression in *Scalindua sp.* were more dominant under ammonium-limiting conditions. 762 genes were either up- or downregulated representing slightly more than 15% of the total predicted ORFs in *Scalindua sp.*. Under nitrate limitation significant changes only occurred in the expression of 228 genes (4.6%), many of which did not show a similar expression pattern in the biological replicate and were therefore not considered in further analyses. This was not surprising as the anammox process was limited in nitrite both under the control condition and nitrate limiting condition. *Sedimenticola sp.* differentially expressed 742 genes under nitrate limitation which represents 17% of all its genes. Ammonium

limitation resulted in the up- or downregulation of 331 genes.

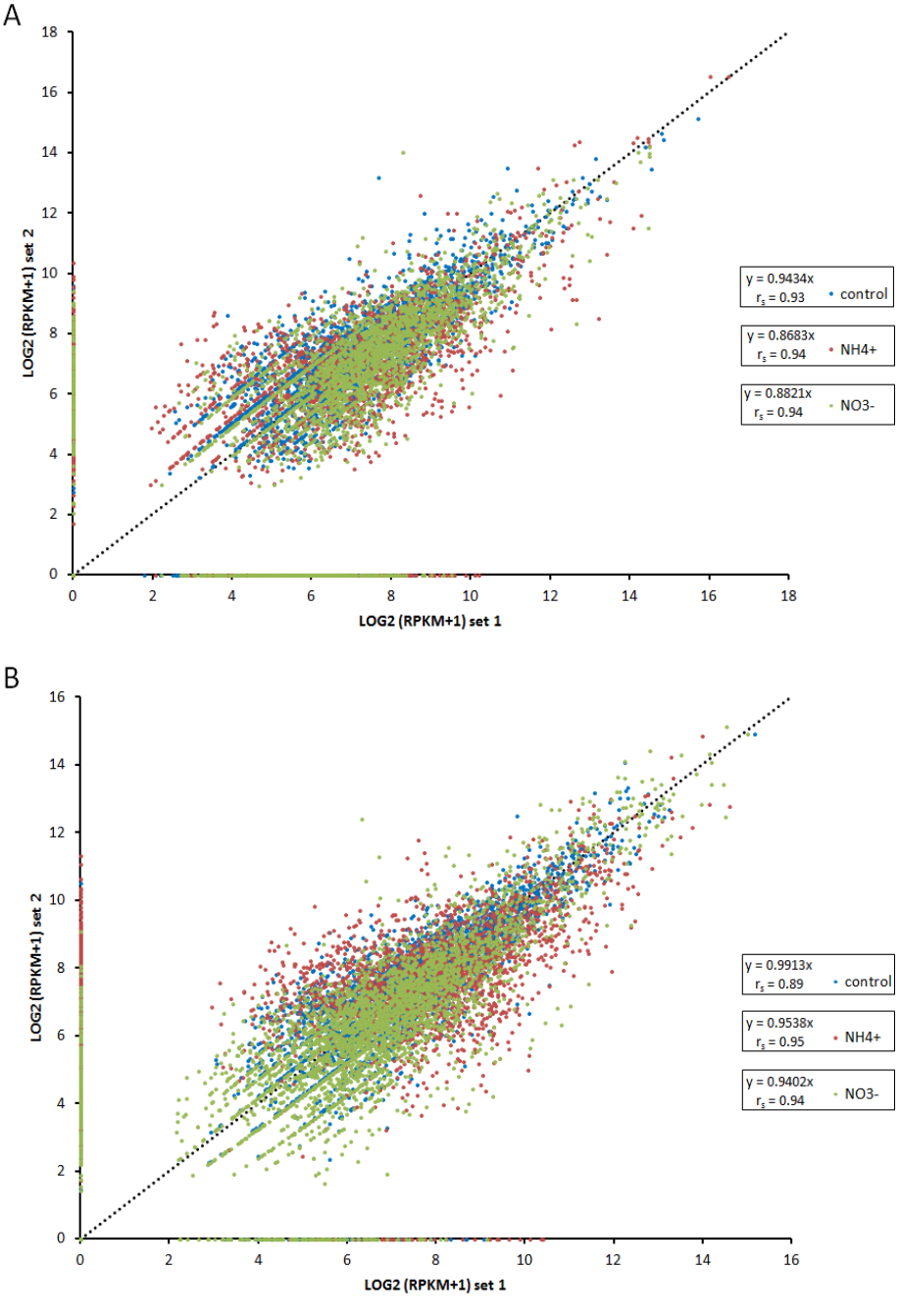

Figure 2: Correlation of LOG2(RPKM+1) values of the duplicate gene expression (*Scalindua sp.* A, *Sedimenticola sp.* B) sets to show reproducibility (Spearman's rank correlation coefficient (rs)). The dotted line indicates x=y for identical gene expression. Colors correspond to the different conditions: control (blue), NH_4^+-limited and NO_3^--limited (green).

Scalindua sp. metabolism under ammonium limitation

Anammox bacteria are autotrophs that gain energy for growth by oxidizing ammonium anaerobically while reducing nitrite. It is a stepwise process in which NO_2^- is first reduced to NO and then combined with NH_4^+ to form hydrazine, which is further oxidized to N_2 (Kartal et al., 2011). The limitation of substrates is a condition that anammox bacteria face regularly in their natural habitats. Especially in marine systems ammonium has been observed to be the limiting substrate (Lam and Kuypers, 2011). But how does anammox respond to a ammonium limitation on a molecular level?

Transporters

The first barrier a potential substrate faces is the bacterial outer membrane, which most of the time requires active transport of the substrate to pass it. Anammox bacteria even have a second internal membrane surrounding the anammoxosome, an organelle-like structure where the energy metabolism takes place (van Niftrik et al., 2004; Kartal et al., 2011; van Teeseling et al., 2013). It therefore requires a whole array of transporters that can get substrates and metabolites to their required destination. *Scalindua sp.* encodes four transporters belonging to the formate/nitrite transporter family (FNT) that are likely to be involved in nitrite translocation. Two of them share the highest similarity with *E.coli nirC* sequences (scal00416 and scal04132) and the other two with formate transport systems *focA* (scal00974/75). One of the *nirC* genes (scal00416) was the most highly expressed FNT family transporter under control conditions, but it was downregulated under NH_4^+ limitation. The second *nirC* gene (scal04132) was not expressed under any of the conditions. The downregulation of the *nirC* transporter could have several reasons: Under control conditions nitrite was not detectable in the effluent, so the uptake of nitrite probably required a high affinity transporter. When ammonium was limiting, nitrite accumulated outside of the cell, possibly requiring a transport system with a different affinity. That would imply that another, lower affinity nitrite transporting system could be active under ammonium limitation. Alternatively, nitrite transport into the cell might be restricted in general, as the shortage of ammonium would slow down downstream anammox reactions. This would suggest a first line regulation of metabolism, preventing a high internal nitrite concentration and the resulting NO poisoning. Comparing expression levels of the expressed *nirC* and the two *focA* genes implicated scal00146 as the likely candidate as the physiologically most important nitrite transporter (Tab. 1). It has to be considered however that nitrite has to pass the cytoplasmic as well as the anammoxosome membrane, which means that

the two different membranes require specific transporters. The *focA*-like genes (scal00974/75) were upregulated under ammonium limitation (set 1: 7-fold/ set2: 11-fold; set 1: 6-fold/set2: 5-fold) (see Tab. 1). It is known that NirC and FocA proteins are not selective for nitrite and formate respectively. Both transporters can translocate both anions, but usually with lower affinity than their dedicated substrates (Lü et al., 2012).

In addition to nitrite, also ammonium needs to be transported into the cell. The analyzed *Scalindua* sp. genome encoded at least 5 different *amtB* ammonium transport systems (van de Vossenberg et al., 2013). Four of the ammonium transporter genes were encoded in a gene cluster and are probably regulated by two P-II regulatory proteins (GlnK) (scal00587-scal00596). Under control conditions the most highly expressed *amtB* gene was scal00587, followed by scal00596 and scal01681 (Tab. 1). When ammonium was limiting *amtB* expression of the *amtB* cluster increased more than 7-fold (Tab. 1). The expression of scal01681 did not change and scal03708 was not expressed under any of the conditions. The most significant upregulation was observed in scal00591 (44/129-fold) and scal00596 (10/16-fold). Based on assumptions by Medema et al. (2010), the *K. stuttgartiensis* analogues to scal00596 and scal01681 would be the only ammonium transporters targeted to the cytoplasmic membrane. The others are likely to be located in the anammoxosome membrane. If that was true then significant upregulation of *amtB* genes targeted to both membranes would make ammonium transport to the anammoxosome possible. The AmtB proteins are commonly linked to P-II regulatory proteins (GlnK) controlling many aspects in nitrogen metabolism (Coutts et al., 2002; Javelle et al., 2004; Boogerd et al., 2011). It is known that GlnK forms a membrane-bound complex with AmtB upon which the transporter is inactivated. Previous studies mainly focused on microorganisms using ammonium as N source, where the internal pools of 2-oxoglutarate (high) and the ATP/ADP ratio (high) cause the dissociation of the complex. The regulation of transporters in aerobic and anaerobic ammonium oxidizers using ammonium for catabolic processes is not resolved, but it is likely to proceed in a similar fashion. The synchronized regulation of *amtB* and *glnK* has been described before when *Dehalococcoides ethenogenes* strain 195 was limited in fixed nitrogen as N-source (Lee et al., 2011). Also in *Scalindua* sp. the *glnK* genes in the *amtB* cluster were highly upregulated under ammonium limitation, probably allowing for a quick response to changing ammonium concentrations.

Table 1: RPKM values of anammox key metabolic genes under differential limitations in duplicate (1,2).

Annotation	Gene	*S. profunda* gene id	RPKM NH_4^+ limited		RPKM control		RPKM NO_3^- limited	
			1	2	1	2	1	2
Hydrazine synthase	*hzsA*	scal01318	91.808	94.674	21.578	18.762	22.928	15.094
Hydrazine synthase	*hzsBC*	scal00025	66.587	95.440	29.466	22.167	18.963	16.769
Hydrazine dehydrogenase	*hdh*	scal03295	6.190	19.483	53.855	35.860	23.004	18.642
Hydroxylamine oxidoreductase	*hao*	scal01317	22.575	22.755	28.620	25.949	19.769	13.372
Hydroxylamine oxidoreductase	*hao*	scal02288	25	296	117	237	135	52
Hydroxylamine oxidoreductase	*hao*	scal04133	358	344	235	444	417	613
Hydroxylamine oxidoreductase	*hao*	scal02110	1.746	1.475	8.083	8.066	9.394	8.789
Hydroxylamine oxidoreductase	*hao*	scal02116	1.940	1.969	1.525	2.321	2.800	2.833
Hydroxylamine oxidoreductase	*hao*	scal00421	2.217	2.350	7.907	5.162	3.928	3.974
cd_1 nitrite reductase	*nirS*	scal02098	1.579	2.518	6.236	5.787	7.822	7.216
Nitrite oxidoreductase	*nxrA*	scal00863	1.588	2.329	8.409	6.842	6.502	5.684
Nitrite oxidoreductase	*nxrB*	scal00867	775	1.200	7.831	5.602	4.025	3.394
formate/nitrite transporter	*nirC*	scal00416	431	523	4.547	4.721	2.438	1.936
Formate efflux transporter	*focA*	scal00974	1208	1006	175	93	108	93
Formate efflux transporter	*focA*	scal00975	511	1113	91	234	140	193
Nitrate/nitrite antiporter	*narK*	scal03007	242	190	198	355	165	151
Ammonium transporter	*amtB*	scal00587	4.559	7.859	2.046	2.505	1.757	2.343
Ammonium transporter	*amtB*	scal00591	6.802	21.075	154	164	51	585
Ammonium transporter	*amtB*	scal00594	849	959	125	134	207	150
Ammonium transporter	*amtB*	scal00596	3.695	7.099	372	438	408	675
Ammonium transporter	*amtB*	scal01681	154	531	495	566	362	334
Hydroxylamine reductase Hcp	*hcp*	scal03703	1.232	769	25	132	122	118

Key genes in catabolism

Once the substrates have entered the cell they need to be converted into useful energy. In anammox bacteria this involves nitrite reduction to NO followed by the condensation of NO and NH_4^+ to produce the unique intermediate hydrazine, which in turn in converted to N_2 (Kartal et al., 2011). Hydrazine synthesis by anammox bacteria is so far unique in nature and is catalyzed by hydrazine synthase (HZS). The gene encoding HZS was the most highly expressed mRNA in the transcriptome and most abundant protein in the proteome of *K. stuttgartiensis* (Kartal et al., 2011). Also in *Scalindua* sp. genes encoding this protein showed the highest expression, but unlike in *K. stuttgartiensis*, where the enzyme consists of 3 subunits (αβγ), genes encoding the β and γ- subunit of this enzyme in *Scalindua sp.* were fused (scal00025) (van de Vossenberg et al., 2013). The *hzs* gene cluster (scal02632-36, scal00025, scal01317-18) encodes a putative heme protein and a cytochrome *c* family protein, which are assumed to be membrane components comprising the electron transfer module (ETM) of the functional enzyme complex (de Almeida, 2014). In this hypothesis, the putative cytochrome *c* (scal02634) could transfer electrons from the membrane bound ETM to the soluble HZS. Other components of the *hzs* gene cluster are a sigma 54-like response regulator (scal02635), the hypothetical protein (scal02636), HzsBC (scal00025), HzsA (scal01318) and the HAO-like protein (scal01317), which is highly expressed (Tab. 1) also in *K. stuttgartiensis* (kustc1061). Short-term ammonium limitation triggered a 4 to 5-fold upregulation of *hzsA* and a 2 to 4-fold increase in *hzsBC*. Conversely, the membrane components scal02632/33 (10/5-fold) as well as the putative electron carrier (scal02634; 4-fold) were downregulated. The putative response regulator (scal02635) changed expression by only 2-fold (downregulation), whereas the protein of unknown function (scal02636) was upregulated (6/7-fold). Expression of the HAO-like protein proposed to be involved in the detoxification of hydroxylamine by recycling it as NO (Maalcke et al., 2014) did not change under any of the conditions (Tab. 1). Hydrazine synthesis was suggested to be the rate-limiting step in the overall anammox reaction under control conditions (Kartal et al., 2011). Under ammonium limitation this bottleneck might become even tighter as the enzyme was not only limited by its own reaction speed, but also by the limited supply of ammonium due to the unfavorable ammonium to nitrite ratio. This might result in an increased transcription of the HZS enzymatic subunits to increase the chance of converting ammonium as efficiently as possible. The downstream process converts hydrazine to dinitrogen gas and is catalyzed by hydrazine dehydrogenase (HDH; scal03295). As less NH_4^+

and NO were condensed to hydrazine the gene expression encoding for this enzyme also decreased. The genome of *Scalindua* sp. harbors at least 7 other *hao*-like proteins. Two of which (scal02110 and scal00421) have been suggested to be able to reduce nitrite to NO (Kartal et al., 2013). This hypothesis was based on sequence analyses and the fact that cd_1 nitrite reductase (NirS) was expressed only at low levels in *K. stuttgartiensis*. *Scalindua* sp. however, highly expressed the typical cd_1-type nitrite reductase (*nirS*). Interestingly, under ammonium limitation, both NirS (3x) and the two HAO-like proteins scal02110 (5x) and scal00421 (2-3.5x) were downregulated. Keeping in mind that under ammonium limitation nitrite derived from nitrate reduction did accumulate, downregulation of the nitrite reduction pathway would make sense as there was too little ammonium to be combined with the end product of nitrite reduction (NO) to form hydrazine. This could imply a downregulation of the *nirS* and *hao* genes to prevent NO-poisoning. The expression of the remaining HAO-like proteins did not change significantly with changing conditions (Tab. 1).

Growth of anammox bacteria is associated with the oxidation of nitrite to nitrate. It was previously assumed that this reaction was necessary to gain reducing equivalents for carbon fixation (van de Graaf., 1996; Schouten et al., 2004). Recent evidence however indicated that this reaction could instead be coupled to the reduction of nitrite to NO (Hu et al., 2014). In agreement with the nitrite-reducing processes (*hao* scal02636 and *nirS* scal02098) also the catalytic subunits of the nitrite:nitrate oxidoreductase cluster (*nxrA* scal00863, *nxrB* scal00867) were downregulated under ammonium limitation. Another interesting gene that was differentially regulated, but does not have a homologue in *K. stuttgartiensis* or KSU-1 is a putative hybrid cluster protein (HCP, scal03703), which was annotated as a hydroxylamine reductase. It shared the highest similarity with *Desulfosarcina* sp. BuS5 (64% similarity on protein level) and was strongly upregulated (50/6-fold) under ammonium limitation (Tab. 1). The physiological role of HCP's is currently unknown, but there are several reports showing their involvement in a nitrosative stress response (Boutrin et al., 2012; Yurkiw et al., 2012). HCP's with *in vitro* hydroxylamine and hydrogen peroxide reduction activities have been characterized as well (Wolfe et al., 2002; Almeida et al., 2006). Its exact function in *Scalindua* sp. is unknown at this point, but as nitrite concentrations are elevated under ammonium limitation it might also be involved in a nitrosative stress response.

Energy conservation

Under conditions of anaerobic ammonium oxidation, ammonium is an

essential substrate for energy conservation. The low ammonium:nitrite ratio supplied therefore lead to the downregulation of genes involved in energy conservation due to the reduction of anammox catabolism. The bc_1 complex has an essential role in anammox energy metabolism by coupling electron transfer to the generation of a proton motive force (*pmf*) that then drives ATP synthesis. The *Scalindua* genes scal02105-10 encode the bc_1 complex homolog with the highest expression in *Kuenenia stuttgartiensis* (kuste4569-74; bc-3) (Kartal et al., 2013; de Almeida et al., 2014). All genes were differentially downregulated in *Scalindua sp.* under ammonium limitation.
Energy conservation in anammox bacteria is driven by the buildup of a *pmf* driving ATP synthesis. Genes encoding the proton-translocating ATP synthase cluster (scal00196- scal00201) were downregulated by 5-fold on average. The homologue of this cluster has been described as the major ATPase in *Kuenenia stuttgartiensis* (van Niftrik et al., 2010) and was also the highest expressed ATPase cluster under control conditions in *Scalindua sp.* Downregulation of the energy conservation pathway was the result of a lack of ammonium, its acquisition representing the bottleneck in anammox catabolism under NH_4^+-limiting conditions.

Nucleotide, RNA and DNA metabolism and cell division
In response to ammonium limitation, an important substrate in the anammox energy metabolism, a lot of genes involved in cell maintenance and growth were downregulated. A large number of transcripts coding for ribosomal proteins and proteins involved in tRNA synthesis were downregulated more than 3-fold or not expressed at all under ammonium limitation. Also RNA metabolism genes were downregulated: 24% of the RAST annotated genes coding for proteins involved in purine and pyrimidine metabolism and 32% of the genes involved in RNA metabolism were downregulated by more than 3-fold. This included key genes such as several subunits of the DNA-directed RNA polymerase (α, β, β') and transcription termination factors (*nusA*, *rho*). Accordingly also genes playing a role in the production of amino acids and protein folding, such as the chaperones DnaJK and GroEL/ES were less expressed. Genes encoding proteins involved in DNA replication were downregulated significantly as 20% of the transcripts of these genes were expressed at least 3 times lower than under control conditions. Also the expression of several cell division genes were downregulated: Two bacterial AAA proteases encoded by *ftsH, ftsK* required for cell division and chromosomes segregation, *mraZ* a cell division protein of unknown function and a cell division trigger factor were expressed less. Other cell division

genes were either not affected or could not be retrieved from the *Scalindua sp.* genome, like an *ftsZ* homolog or its potential *K. stuttgartiensis* ortholog kustd1438 (Van Niftrik et al., 2009).

Motility

Motility is an important characteristic enabling microorganisms to escape less favorable conditions. Many bacteria produce appendages (i.e. flagella, pili) that are involved in active movement under certain conditions. Although in anammox these cell appendages could only be observed sporadically by electron microscopic imaging of anammox bacteria under standard growth conditions (van Niftrik et al., 2008), the genomes of *K. stuttgartiensis* and *Scalindua sp.* harbor all the necessary genes to make flagella and possibly other filaments (Strous et al., 2006; van de Vossenberg et al., 2013). Nutrient limitation is one of the factors that can lead to the expression of a flagellum (Adler and Templeton, 1967; Byrne and Swanson, 1998) and accordingly lots of flagellar genes are upregulated more than 10-fold under ammonium limitation. Flagellum assembly has been studied well for a handful of model organisms and an extensive diversity has been described, making it difficult to deduce a mutual mechanism (McCarter, 2006; Liu and Ochman, 2007). Presumably due to the short duration of the exposure to ammonium-limiting conditions the expression of early flagellar genes leading to MS- and C-ring assembly and the proximal rod were highly expressed. Also the anti-sigma factor FlgM was highly represented in the transcriptome (30/7-fold upregulated). This protein controls late gene expression by targeting the RNA polymerase sigma factor until the flagellar export apparatus is capable of FlgM secretion (McCarter, 2006). As the internal concentration of FlgM drops, the RNA polymerase sigma factor is released and initiated expression of late flagellar genes. At the moment of transcriptome sampling the FlgM target shows little expression yet, resulting in a low number of transcripts of late gene products within the flagellar operon. This implies that the flagellum is in early stage of assembly.

Carbon fixation

Anammox bacteria fix CO_2 via the Wood-Ljungdahl pathway (Schouten et al., 2004; Strous et al., 2006), where CO_2 is first reduced to CO and consequently to acetyl-CoA catalyzed by a bifunctional cluster of the two enzymes: CO dehydrogenase and acetyl-CoA synthase (CODH/ACS). The putative physiological CO_2-fixing CODH/ACS complex encoded by scal02484-89 was downregulated under ammonium limitation. This cluster included one

of the catalytic subunits (*acsB*) as well as *acsCFD* and a putative ferredoxin-like protein belonging to the same cluster. As the *Scalindua sp.* genome is still fragmented the *acsA* subunit (scal02114) was only partial and present on a different contig and was highly expressed under all conditions. Generally the carbon fixation pathway was affected by ammonium limitation, possibly a response to a lack of energy.

Nitrogen assimilation

The assimilation of nitrogen often starts with ammonium and proceeds via glutamine and glutamate. There are two different pathways of ammonium assimilation: a high affinity pathway in which glutamate dehydrogenase (GDH) produces glutamate by the reductive amination of 2-oxoglutarate and a low affinity pathway involving the two enzymes glutamine synthase and glutamate synthase (GS-GOGAT) which first produce glutamine that is in turn transformed to glutamate by transfer of the amide group of glutamine to 2-oxoglutarate (Yan, 2007; Gunka and Commichau, 2012). Under control conditions *Scalindua sp.* expressed both systems with the GDH pathway showing approximately 2-fold higher expression. It would seem that also in anammox bacteria the glutamate dehydrogenase pathway assimilated ammonium for cellular nitrogen under high ammonium concentrations, as a downregulation (4/5-fold) of the gene was observed under ammonium limitation. Accordingly the GS-GOGAT pathway was upregulated when ammonium was limiting. A putative glutamine synthase (scal00246) was upregulated by 6/2-fold and the glutamate synthase large and small subunits (scal01730/1) were upregulated by 2-fold. It has been stated earlier that the GS-GOGAT pathway is more suitable for ammonia assimilation under N-limitation, as GS has a much lower K_m for ammonia than GDH (Reitzer, 2003). However, it cannot be stated with certainty that the gene for GDH is also functional in ammonia assimilation as several microorganisms use it for glutamate degradation only (Gunka and Commichau, 2012).

Sedimenticola sp. and substrate limitation

Sedimenticola sp. was described as strictly anaerobic, non-motile rod that can use either nitrate, nitrite or selenate as electron acceptor while oxidizing a number of organic compounds (Narasingarao and Häggblom, 2006). Later it was shown that also reduced sulfur species were used electron donor by this microorganism (Russ et al., 2014). Ammonium was not a substrate of *Sedimenticola sp.* and we therefore assumed that nitrate limitation would have the highest impact on the differential expression of genes in *Sedimenticola sp.*

as our results also showed.

Denitrification

The first step in denitrification is the reduction of nitrate to nitrite catalyzed by a nitrate reductase. *Sedimenticola sp.* possesses both a respiratory (*nar*) and a periplasmic (*nap*) nitrate reductase cluster. The differential regulation of these two systems in this bacterium is unknown, but in other microorganisms nitrate concentrations can influence the expression (Wang et al., 1999; Stewart et al., 2002). The *nar* cluster was downregulated under NO_3^- limitation. The genes encoding the α, β- and δ-subunit were downregulated by more than 20-fold compared to the control condition whereas the gene encoding the γ-chain was downregulated more than 5-fold. Also two nitrate/nitrite transporters and a nitrate/nitrite sensor protein encoded in the same gene cluster were downregulated. The differential expression of the periplasmic nitrate reductase (*nap*) was not clear as the biological replicates provided conflicting results (no downregulation in set 1/ 8-fold downregulation in set 2) (Tab. 2). However, the ratio of the catalytic subunits *narG*:*napA* changes under NO_3^- limitation with *napA* being relatively higher expressed (9/80-fold). This would make sense as the periplasmic nitrate reductase has been described to be active under lower nitrate concentrations (Richardson et al., 2001). Both nitrate reductase clusters were also differentially downregulated by more than 5-fold when NH_4^+ was limiting. The *nar* operon is regulated by a two component transcriptional regulator of the LuxR family, *narXL* located upstream of the cluster. A sensory system detects the ratio of nitrate:nitrite and deactivates NarL when nitrite is high, thereby downregulating the expression of the *nar* operon and possibly also the *nap* operon (González et al., 2006). As the nitrite concentration increased upon ammonium limitation, nitrate reductase expression was downregulated. In *E. coli* the use of nitrate as terminal electron acceptor and the expression of the nitrate reductase gene is closely coupled to formate dehydrogenase, which is also regulated by NarL (Berg and Stewart, 1990; Wang and Gunsalus, 2003). Under anaerobic conditions formate dehydrogenase oxidizes formate to CO_2 in the periplasm and transfers the electrons via the quinone pool to proteins for the reduction of respiratory substrates. Under denitrifying conditions the electrons are shuttled to nitrate reductase to subsequently reduce nitrate in the cytoplasm thereby establishing a proton gradient. In *Sedimenticola sp.* a gene cluster similar to the formate dehydrogenase N of *E. coli* could be detected (WP_029133272-76). It was in line with the previously observed regulation pattern as all subunits of the complex were significantly downregulated (3-

21-fold) under nitrate-limiting conditions corroborating the downregulation of the *nar* cluster. The enzyme was highly expressed under control conditions indicating an important function in *Sedimenticola sp.*.

The cd_1-type nitrite reductase (*nirS*), catalyzing the second step in denitrification was not expressed at the same level in the biological replicate under nitrate limitation (1.3-/8-fold) (Tab. 2). *Sedimenticola sp.* responded to elevated nitrite concentrations under ammonium limitation however by upregulating the *nirBD* gene cluster (WP_029134437/38) by 14/75-fold. These genes encode an enzyme that reduces nitrite to ammonia and might thereby detoxify nitrite and also make it available as alternative N-source under ammonium limitation (Wang and Gunsalus, 2000; Luque-Almagro et al., 2011). Downstream of this cluster another nitrite/nitrate transporter is located, which was also upregulated under NH_4^+ limitation (8/11-fold).

The last steps in denitrification involve the reduction NO to N_2O by nitric oxide reductase (*norB*) and consequently the conversion of N_2O to N_2 by nitrous oxide reductase (*nosZ*). Although the previous study could show that the majority of nitrate reduced is released as nitrite and that only around 35-25% of the N_2 is released by denitrification (Russ et al., 2014), both genes were highly expressed under control conditions (Tab. 2). Genes composing the catalytic subunit of the nitrous oxide reductase (*nosZ*) did not change significantly under any of the conditions (Tab. 2). The NO reductase (*norB*) however was downregulated under nitrate limitation, but several of its regulatory genes were upregulated.

Sulfur metabolism

Under nitrate limitation the absence of a suitable electron acceptor resulted in the downregulation of many genes involved in sulfur metabolism that were highly expressed under control conditions (Tab. 2): All *sox* genes (>5-fold), sulfide dehydrogenase (>10-fold) and adenylylsulfate reductase (>3-fold). The last step in sulfide oxidation is the conversion of adenylyl sulfate to sulfate catalyzed by the enzyme sulfate adenylyltransferase (*sat*). This gene is upregulated (>3-fold) under nitrate limitation.

Energy conservation and core metabolism

Also in *Sedimenticola sp.* the lack of suitable substrates resulted in the downregulation of many genes involved in central metabolism and energy conservation. In short, substrate limitation affected all ATP synthase subunits (downregulation), the TCA cycle was downregulated including enzymes such as malate dehydrogenase, succinyl-CoA ligase, pyruvate dehydrogenase. Protein

Table 2: RPKM values of dentrifier key metabolic genes under differential limitations in duplicate (1,2).

Annotation	Gene	*S. selenatireducens* gene id	RPKM NH_4^+ limited		RPKM control		RPKM NO_3^- limited	
			1	2	1	2	1	2
Periplasmic nitrate reductase	*napA*	WP_029134337	196	188	1.838	909	1.694	112
Nitrate reductase	*narG*	WP_029132616	260	60	893	476	21	13
cd_1 nitrite reductase	*nirS*	WP_029134679	2.353	3.170	7.105	5.124	5.333	617
NO reductase	*norB*	WP_029133259	486	622	905	744	105	123
Nitrous oxide reductase	*nosZ*	WP_029133254	4.094	3.601	8.942	5.615	4.502	9.539
Sulfide dehydrogenase (cytochrome)	*fccA 1*	WP_029133266	2.467	2.365	5.809	3.676	352	209
Sulfide dehydrogenase (cytochrome)	*fccA 2*	WP_029133834	385	1.067	2.684	1.475	317	264
Sulfide dehydrogenase (flavoprotein chain)	*fccB 1*	WP_029133267	2.680	2.860	5.590	2.993	265	73
Sulfide dehydrogenase (flavoprotein chain)	*fccB 2*	WP_029133835	791	623	1.841	714	90	72
Sulfur oxidation protein	*soxY 1*	WP_029131896	608	1.222	1.290	1.071	431	244
Sulfur oxidation protein	*soxY 2*	WP_029132147	213	2.741	1.124	1.030	104	59
Sulfur oxidation protein	*soxZ 1*	WP_029131895	5.090	1.306	2.028	1.541	683	146
Sulfur oxidation protein	*soxZ 2*	WP_029132148	0	2.525	1.418	1.117	32	54
Sulfur oxidation protein	*soxB*	WP_029132149	113	383	174	152	0	12
Sulfite reductase	*dsrA*	WP_029131904	3.863	4.992	2.009	1.187	2.253	1.358
Sulfite reductase	*dsrB*	WP_029131905	2.799	5.535	1.921	1.295	1.046	1.003
Adenylylsulfate reductase	*aprA 1*	WP_029133846	1.017	2.077	1.882	1.446	517	121
Adenylylsulfate reductase	*aprA 2*	WP_029134892	7.536	5.695	8.196	5.721	2.235	2.121
Adenylylsulfate reductase	*aprB 1*	WP_029133847	1.724	2.380	1.178	905	213	178
Adenylylsulfate reductase	*aprB 2*	WP_029134891	2.041	2.102	4.012	3.169	6.057	5.758
Sulfate adenylyltransferase	*sat*	WP_029134400	1.033	836	937	871	4.525	2.935

metabolism was affected by the downregulation of transporters involved in amino acid translocation and enzymes involved in the conversion of amino acids, but also by the downregulation of genes encoding transcription- and translation- relevant enzymes and genes with an essential role in cell division (*ftsZAI*). Additionally the NADH-ubiquinone oxidoreductase subunits of complex I were downregulated possibly affecting the respiratory chain by interrupting the generation of a proton gradient which is directly linked to ATP generation.

In the reactor system used in these experiments *Sedimenticola sp.* grew autotrophically by fixing CO_2 via the Calvin-Benson-Bassham (CBB) cycle. The genome of *Sedimenticola sp.* harbors 2 gene copies encoding the ribulose bisphosphate carboxylase (Rubisco) catalyzing the first and essential step in CO_2 fixation via this pathway: A classical type I Rubisco forming hexadecameric protein structure consisting of a large (WP_029133841) and a small (WP_029133840) subunit and a type II Rubisco (WP_029132057) consisting of multimers of only the large subunit. The gene encoding the type II Rubisco was significantly higher expressed than type I under all conditions (>4-fold). Type II Rubisco has a low O_2 discrimination threshold, a poor affinity for CO_2, and a high k_{cat} compared to type I Rubisco (Watson and Tabita, 2006; Badger and Bek, 2008). These kinetic properties make it an ideal candidate for fixing CO_2 in microaerobic or anoxic environment with high CO_2 concentrations, the exact conditions in our reactor system. Under nitrate limitation type II Rubisco was upregulated by 3/6-fold.

Nitrogen assimilation

Comparing the expression levels of the assimilation pathways of nitrate and ammonium, ammonium seems to be the preferred N-source for *Sedimenticola sp.*. Under ammonium limitation both *amtB* ammonium transporter genes were highly upregulated (10-40x). The imported ammonia is probably assimilated in the GS-GOGAT and not the GDH pathway, as the glutamate dehydrogenase was not expressed at high levels. Both subunits of the glutamate synthase were not differentially expressed under the conditions tested. However glutamine synthase representing the first step in ammonium assimilation by catalyzing the condensation of glutamate and ammonia to form glutamine was upregulated by 10-/7-fold. This indicated that *Sedimenticola sp.* was trying to scavenge residual ammonium for cellular nitrogen, entering the competition with anammox for substrates. As mentioned earlier, *Sedimenticola sp.* might also use nitrite as an additional N-source under ammonium limitation by reducing nitrite to ammonia by nitrite reductase (*nirBD*).

Experimental procedures

Analytical methods

Nitrite was measured colorimetrically at 540 nm after a 5 min reaction of 1 ml sample (0.1 – 0.5 mM nitrite) with 1 ml 1% sulfanilic acid in 1 M HCl and 1 ml 0.1% naphtylethylene diaminedihydrochloride. Ammonium was measured at 420 nm on a Cary Eclipse Fluorescence Spectrophotometer after reaction with 10% ortho-phthaldialdehyde as has been described previously (Taylor et al., 1974). Nitrate was measured by converting nitrite and nitrate in the samples to nitric oxide in a saturated solution of VCl_3 in 1M HCl at 95 °C. NO was then measured by the Nitric Oxide Analyzer NOA280i (GE Analytical Instruments, USA) according to the protocol.

Reactor operation

The previously described fermentor (Russ et al., 2014) containing a co-culture of anammox bacteria and the sulfur-oxidizing *Sedimenticola* sp. was used to gradually decrease the ammonium concentrations by lowering the feed from influent concentrations of 9 mM nitrate and 7 mM ammonium to 1 mM ammonium. Ammonium concentrations were limiting when nitrite started accumulating. Cells were harvested for molecular analysis and the influent ammonium concentrations were restored to 7 mM. In a second step influent nitrate concentrations were lowered to 1 mM. The nitrate concentrations in the reactor were followed and cells were harvested when the concentration was below the detection limit (200 nM). These experiments were performed twice 08/13/2013-09/02/2013 and 01/20/2014-02/20/2014 to obtain a biological replicate.

RNA isolation and library preparation

RNA was extracted from 12 ml co-culture biomass using the TRIzol® Reagent (Life Technologies, USA) following the protocol. Concentrations were determined spectrophotometrically using a NanoDrop (Thermo Scientific). 10 µg total RNA was used in the MICROBExpress™ kit (Life Technologies, USA) to enrich bacterial mRNAs by specifically removing 16S and 23S rRNA. The sample was eluted in 25 µl nuclease free water. An additional cleaning step was needed to clean the sampled from 5S rRNA using the MEGAclear™ Kit (Life Technologies, USA). 24 µl of sample from the previous purification step was used and the RNA quantity was checked on the NanoDrop and 2100 Bioanalyzer (Agilent Technologies, USA) yielding 170 to 220 ng mRNA per sample. Further library preparation was performed

using the Ion Total RNA-Seq Kit v2 for whole transcriptome libraries (Life technologies, USA) incubating the sample with RNAse III for 7 min and further following manufacturer's instructions. Emulsion PCR was done using the Onetouch 200 bp kit and sequencing was performed on an IonTorrent PGM using the Ion PGM 200 bp sequencing kit and an Ion 318 chip (Life technologies, USA).

Data analysis

Analysis of reads was performed using CLC genomics workbench 7.0.4 (CLCbio, Denmark). The reads were length and quality score trimmed (>50 bp, 0.05) resulting in datasets that can be found in Supplementary Tab. 1. After trimming reads were submitted to RNAseq analysis included in the CLCbio program (Length fraction 0.5, similarity fraction 0.8) using the genome of *Scalindua sp.* (van de Vossenberg et al., 2013) and *Sedimenticola sp.* DSM 17993 as templates. For further comparative analysis the cutoff was set to threefold up- or downregulation of genes compared to the control condition and a minimum of 20 reads matching a gene in at least one of the datasets. Only those genes that showed similar results in the biological replicates were considered.

Supplementary table 1: Details on sequencing reads

Sample	Number of reads	Average length (bp)
Normal 1	1.327.707	112
Normal 2	2.061.064	97
NH_4^+ 1	1.793.834	112
NH_4^+ 2	1.868.186	91
NO_3^- 1	2.113.338	132
NO_3^- 2	2.932.699	95

Supplementary table 2: Reads mapped to the corresponding organsisms (Length fr. 0.5, similarity fr. 0.8)

	Control 1	Control 2	NH_4^+ 1	NH_4^+ 2	NO_3^- 1	NO_3^- 1
Sc. profunda	97.664	46.193	118.547	48.838	89.102	45.793
Se. selenatireducens	102.322	70.480	39.917	92.726	54.779	180.972

Chapter 4

Chapter 5

Amino acids as source of ammonium for *Kuenenia stuttgartiensis*-dominated anammox cultures

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Summary

Anammox bacteria strongly depend on N-cycle transformations by microorganisms for the supply of their substrates. Aerobic ammonium oxidation and nitrate reduction are the most important sources for nitrite. Ammonium can either be supplied via DNRA or remineralization processes. Amino acids are common products of protein remineralization and could potentially be used by anammox bacteria under ammonium limitation. They could serve as additional electron donor and/or be a direct source of ammonium via deamination. Here we investigated the effect of two different modes of amino acid addition (daily pulse-feeding and continuous feeding) on an enrichment of the anammox bacterium *Kuenenia stuttgartiensis*. We monitored nutrient concentrations and used metagenomics to determine changes in the microbial community composition. The addition of amino acids stimulated nitrate and nitrite reduction rates and led to increasing ammonium concentration over time. As a result the initially ammonium-limited reactor became nitrite-limited, inducing a competition between anammox and other microorganisms for nitrite. Experiments with ^{15}N-labelled glutamate and glycine under ammonium-limitation showed an increase in $^{29}N_2$, indicating that amino acids can serve as a source of NH_4^+ in the anammox process. Metagenomic analysis revealed that amino acid addition supported growth of denitrifying and/or fermentative community members, which accounted for about 40-50% of the reads and was dominated by *Proteobacteria* and *Bacteriodetes*. The stable cooperation between heterotrophic denitrification and anammox could be important in dynamic nitrate-rich systems where fluxes of organic substrates are high.

Introduction

The anaerobic oxidation of ammonium (anammox) is a stepwise process in which nitrite is first reduced to NO and then combined with ammonium to form hydrazine, which is further oxidized to release dinitrogen gas (N_2) (Kartal et al., 2011). So far, the only organisms known to perform this reaction are bacteria belonging to the recently proposed order '*Candidatus* Brocadiales' within the phylum Planctomycetes (Jetten et al., 2010). Since their discovery in a wastewater treatment plant in the Netherlands (Mulder et al., 1995) they have been shown to play a key role in the N-cycle, not only in man-made habitats, but also in numerous natural ecosystems, such as marine oxygen minimum zones (OMZs), anoxic basins, estuaries and peat soils (Dalsgaard et al., 2003; Kuypers et al., 2003; Nicholls and Trimmer, 2009; Hu et al., 2011; Jensen et al., 2011).

In almost all of these habitats anammox bacteria thrive at the oxic/anoxic interface where ambient concentrations of nitrite and ammonium are commonly low. Therefore they depend on other N-cycle transformations for the supply of substrates. Microbial pathways releasing nitrite and thereby supporting the anammox process can be either the (micro)aerobic oxidation of ammonium or nitrate reduction. The significance of these processes as a nitrite source has been investigated in marine environments and under laboratory conditions (Lam et al., 2009; Yan et al 2012 Russ et al., 2014). However, it has been shown that ambient nitrite concentrations often exceed ammonium concentrations and that ammonium is more likely to be the limiting substrate in many marine ecosystems (Lam and Kuypers, 2011).

In the absence of oxygen, biological sources of ammonium can be either remineralization processes that occur through reactions such as heterotrophic denitrification, fermentation, sulfate reduction and methananogenesis or the dissimilatory nitrate reduction to ammonium (DNRA) using various electron donors to reduce nitrate via nitrite to ammonium. Whereas DNRA was suggested to be the major source of ammonium for anammox bacteria in the Peruvian OMZ (Lam et al., 2009), heterotrophic denitrification might play a more important role in the Arabian Sea (Ward et al., 2009). The electron donor for both, denitrification and DNRA, can be organic compounds, which are then mineralized into inorganic molecules such as CO_2, PO_4^{3-} and/or NH_4^+ (Zehr and Ward, 2002). A proportion of organic matter in many ecosystems exists in the form of amino acids (Van Mooy et al., 2002; Zehr and Ward, 2002; Aluwihare and Meador, 2008), which are liberated during the degradation of organic matter. Amino acids are rapidly consumed in the presence and absence of oxygen (Rodger Harvey et al., 1995) and could add

significantly to the ammonium pool. Amino acids might act on the recycling of fixed nitrogen to N_2 via different routes. First, amino acid oxidation could be coupled to denitrification (Van Mooy et al., 2002). This is a remineralization process which would lead not only to nitrate reduction and the release of N_2, but at the same time also to the accumulation of NH_4^+ derived from the amine group of an amino acid. This in turn could be oxidized further with NO_2^- to N_2 by anammox bacteria. The second pathway is hypothetical and based on the ability of anammox bacteria to utilize organic acids i.e. acetate, propionate or formate to reduce nitrate to nitrite and ammonium and combine these two intermediates to N_2 (Kartal et al., 2007). Amino acids might also serve as an electron donor for nitrate reduction in anammox bacteria, but this has not yet been studied. There might even be a third possibility how amino acids could influence the recycling of fixed N directly: Assuming anammox bacteria have the ability to take up and metabolize amino acids they might cleave off the amine group and combine it with NO to form first hydrazine and then N_2. In the two cases that anammox bacteria either use the amino acid as electron donor or as a direct source of ammonium, they might have the additional advantage of being able to use the carbon backbone, which could be used for the production of biomass superseding the need to fix CO_2.
To investigate the effect of amino acids on an ammonium-limited culture of the model anammox bacterium *Kuenenia stuttgartiensis* two different approaches were followed: Daily pulse-feeding a mixture of amino acids (serine, glycine, glutamate) for the course of two weeks and monitoring nutrient concentrations as well as metagenomics to determine changes in the microbial community. The second approach involved the continuous feeding of a *K. stuttgartiensis* enrichment culture with glutamate for 110 days employing the same analytical tools. The combination of these data provided the first insight into the utilization routes of amino acids by an anammox culture, potentially with a denitrifying partner.

Results and Discussion

Genes involved in amino acid degradation
A direct conversion of free amino acids by the anammox bacterium *Kuenenia stuttgartiensis* requires transport of the substrate into the cell. Genome analysis revealed a sodium:dicarboxylate symporter (kuste2738) which might be able to transport glutamate (Mancusso et al., 2012). Another candidate gene that might be involved in the transport of amino acids is kustd1351, which is similar to amino acid permease family proteins in *Peptoclostridium difficile* (40% on protein level), but was also only moderately related (68%) to a similar

gene in *Jettenia caeni* (KSU1_C0346). Homologs to other known amino acid or oligopeptide transporters appeared to be absent in *K. stuttgartiensis*. The genetic potential for the transport of amino acids was therefore difficult to assess and although *K. stuttgartiensis* is likely to be able to transport amino acids, it remains unclear which amino acids can be taken up under which conditions.

Similarly, it was not directly apparent which genes in *K. stuttgartiensis* could be involved in amino acid deamination. To make ammonium available for the anammox reaction the amine group of an amino acid has to be removed and converted to ammonia. This reaction can be performed by amino acid oxidases, which perform the oxidative deamination of an amino acid into its corresponding keto acid, ammonia and hydrogen peroxide (Geueke and Hummel, 2002). As the enzyme requires oxygen it was not surprising that a gene encoding such a protein was absent from *K. stuttgartiensis*. However a gene annotated as hypothetical protein (kuste2470) was related to amine oxidases of Gammaproteobacteria (48%); still, its function in amino acid metabolism remained unclear.

Another class of enzymes facilitating the release of ammonia from amino acids are amino acid dehydrogenases, which use NAD^+ as electron acceptor to oxidize an amino acid into a keto acid and NH_3 in a reversible reaction (Seah, 2007). One of the key enzymes that can be found in all three domains of life is glutamate dehydrogenase, which has an important role in nitrogen assimilation by converting ammonia into glutamate (Reitzer, 2003). This reaction is reversible and can therefore take part in amino acid degradation as well. This would yield the important citric acid cycle intermediate α-ketoglutarate, which could either enter the respiratory chain or serve as precursor for a number of amino acids. *K. stuttgartiensis* also encoded a glutamate dehydrogenase (kustc0555) and an additional truncated copy with a C-terminus similar to a glutamate racemase (kuste3038) an enzyme that is essential in the biosynthetic pathway of the cell wall (Lundqvist et al., 2007). This gene had rather low similarity to glutamate dehydrogenases of the *Thermoanaerobacterales* (27% on protein level) and its role remained unclear. Additionally, *K. stuttgartiensis* also encoded a putative amino acid dehydrogenase (kustc1228) with similarity to *Shewanella* sp. leucine dehydrogenases (57%). Such proteins degrade or synthesize leucine to/from 4-methyl-2-oxopentanoate. Also glycine, if taken up, could serve as a source of ammonium, by the reversible oxidation of glycine with tetrahydrofolate and NAD^+ to CO_2, NH_3, 5,10-methylenetetrahydrofolate and NADH by the glycine cleavage system. This complex consists of the P, H, T and L proteins,

which are all present in *K. stuttgartiensis*. These results indicated that amino acid use in *K. stuttgartiensis* in particular, but most likely also other anammox bacteria could be possible.

Reactor operation during pulse-feeding

To determine the effect of amino acids on a *K. stuttgartiensis* enrichment culture and whether these compounds could be used as a potential source of ammonium, the reactor was first stabilized under ammonium-limiting conditions. The influent contained 25.9 mM of nitrite and 18.5 mM of ammonium corresponding to a load of 7.8 mM $NO_2^-\cdot d^{-1}$ and 5.6 mM $NH_4^+\cdot d^{-1}$. 210 μM of each amino acid (glutamate, glycine and serine) was added daily for 2 days. Before the addition of amino acids the *K. stuttgartiensis* culture consumed 1.3 mol of NO_2^- for every 1 mol of NH_4^+, which was in agreement with the previously described stoichiometry (Strous *et al.*, 1999). Upon the addition of amino acids on day 1, the nitrite concentration remained constant during 8 h of measuring, pointing to an acclimatization phase (Fig. 1). During this phase amino acids were probably taken up, but there seemed to be a lag phase for the liberation of NH_4^+ via deamination. Ammonium concentration stayed below the detection limit (20 μM), but nitrate concentration decreased gradually (Fig. 1).

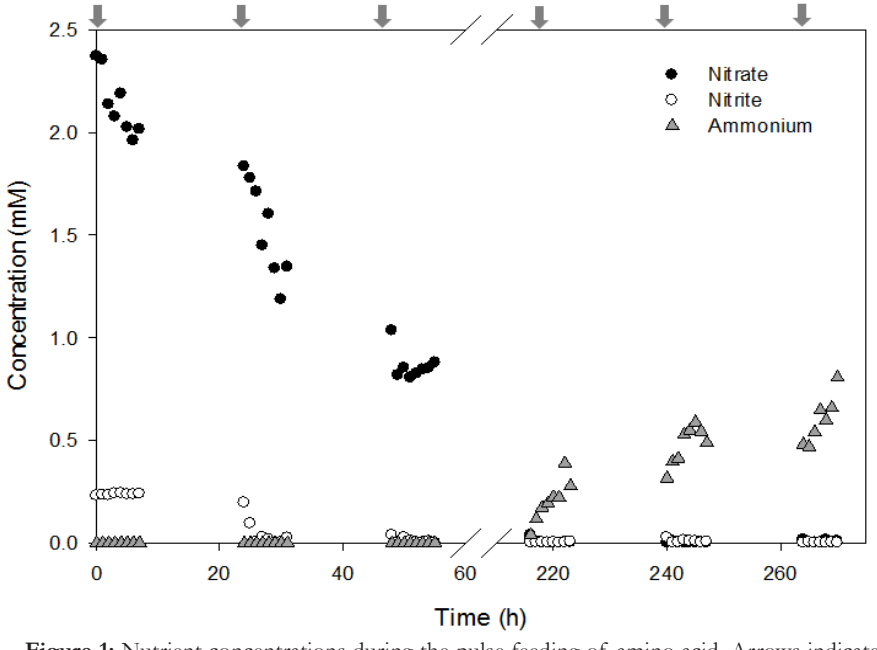


Figure 1: Nutrient concentrations during the pulse feeding of amino acid. Arrows indicate amino acid additions.

There were two different possible routes of nitrate reduction: (1) The dissimilatory nitrate reduction to ammonium (DNRA), which is performed by several groups of microorganisms including the anammox bacteria (Kartal et al., 2007) or (2) denitrifying microorganisms could reduce nitrate to nitrite, and possibly further to NO, N_2O and N_2 using the amino acids as electron donors. In both scenarios anammox bacteria could be supplied with nitrite and ammonium could also be produced.

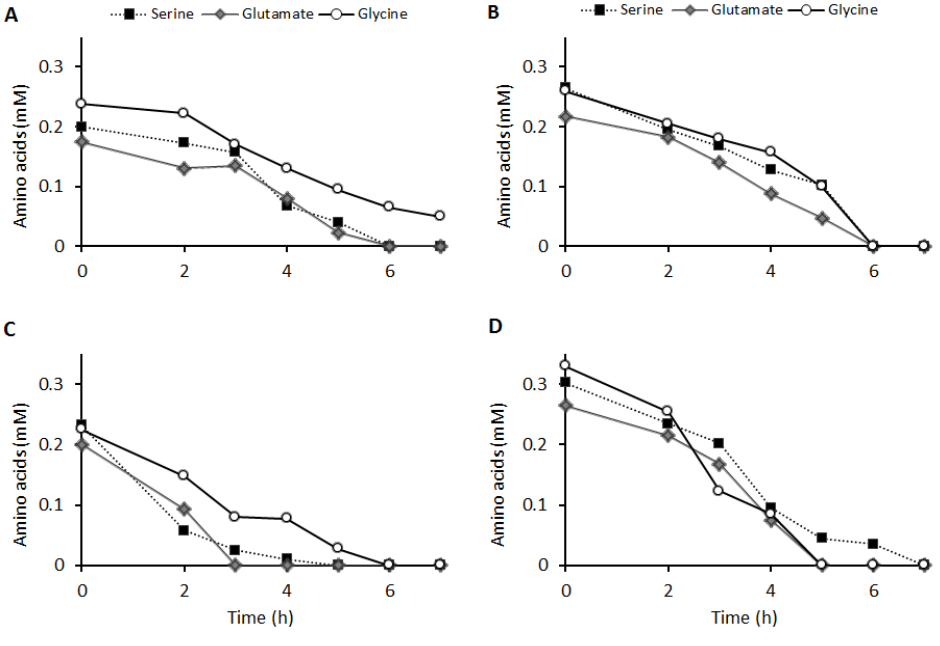


Figure 2: Amino acid concentrations during day 2 and 3 (A,B) and day 10 and 11 (C,D) of amino acid pulse- feeding.

On day 2 and day 3 nitrite concentration dropped rapidly to below 5 µM (detection limit) and nitrate steadily decreased, whereas ammonium concentration remained below the detection limit (Fig. 1). Comparing these data with the amino acid concentrations in the medium (Fig. 2, A&B) suggested that the uptake rates of all three amino acids exceeded their oxidation rates, as nitrate and nitrite reduction continued overnight whereas amino acids were no longer detected after 6-8h. After 10 days of pulse-feeding a mixture of amino acids, nitrite was completely consumed and nitrate concentration in the reactor gradually decreased and stayed below the detection limit (< 200nM) after day 10 (Fig. 1). The absence of nitrate coincided with ammonium accumulation in the reactor and suggested that there was not enough electron acceptor present for anammox bacteria to consume all of the supplied ammonium. As

ammonium started to accumulate after the depletion of nitrate rather than nitrite only, it was likely that nitrite was produced in the reactor through partial denitrification with amino acids. Considering deamination as a potential extra source of ammonium, the ratio of substrate supplied via the medium shifted from 1 mol NH_4^+:1.4 mol NO_2^- to 1 mol NH_4^+:1.25 mol NO_2^-. As the determined stoichiometry of the reactor system before the addition of amino acids was 1.3 mol of NO_2^- for every 1 mol of NH_4^+, additional ammonium would be expected to accumulate immediately if nitrite production was not sufficient. Another observation was the increasing uptake rate of amino acids from the medium from the first to the second week: On day 2 and day 3 after the start of amino acid addition the average uptake rate of glycine, glutamate and serine was 36 ± 4 $\mu M \cdot h^{-1}$ and on day 10 and day 11 this rate increased to 54 ± 11 $\mu M \cdot h^{-1}$ (Fig. 2). There did not seem to be a preference for any of the supplied amino acids because their uptake rates were very similar.

^{15}N tracer experiments

To show the relative contribution ammonium produced via amino acid oxidation to anammox activity, ^{15}N-glutamate or ^{15}N-glycine were used as substrates in separate incubations in an amino acid-adapted culture. Amino acids were added under continuous-culturing conditions with a sealed headspace. In both cases $^{29}N_2$, an indicator of anammox activity, was produced albeit at a relatively low rate of 28 $nM \cdot h^{-1}$ (Fig. 3A & 3B). This rate was due to the low availability of electron acceptors, which resulted in low rates for both anammox ($^{28+29}N_2$) and denitrification ($^{28}N_2$) under nitrite and nitrate limitation. Still this showed that both amino acids served as a source of ammonium at similar rates.

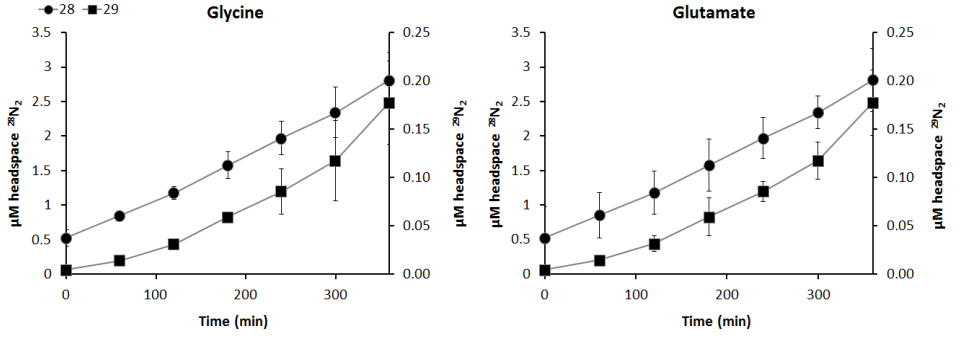


Figure 3: $^{28}N_2$ and $^{29}N_2$ production of a batch-operated reactor system with added ^{15}N-glycine or ^{15}N-glutamate.

Reactor operation during continuous feeding

First the reactor was first stabilized under ammonium-limiting conditions. The influent contained 24 mM of nitrite and 18 mM of ammonium corresponding to a load of 7.2 mM $NO_2^-\cdot d^{-1}$ and 5.4 mM $NH_4^+\cdot d^{-1}$ After a stabilization phase (23 days), the reactor consumed between 5.7 and 5.8 mM $NO_2^-\cdot d^{-1}$ and 5.4 mM $NH_4^+\cdot d^{-1}$ resulting in the stoichiometric consumption of 1.1 mol of nitrite per 1 mol of ammonium. Starting from day 23 glutamate was added to the reactor from a sterile 10.5 mM stock at a flow rate of 100 $ml\cdot d^{-1}$. As a result of glutamate addition, nitrite concentration in the reactor immediately decreased from 1.4 mM to 0.1 mM and then further to below detection limit after day 30, while residual glutamate could be measured (day 29, 250 μM).

The rapid decrease was most likely the result of nitrite reduction by both denitrification and anammox. Interestingly, the depletion of residual nitrite coincided with the appearance of ammonium in the system even though nitrate was still present suggesting that there was no available electron donor to reduce the remaining nitrate to nitrite, which was in line with the observation that the glutamate concentration in the reactor stayed below detection limit (<0.5 μM) after day 30. The addition of amino acids shifted the ratio of nitrite to ammonium from 1.4:1 during the startup phase to 1.23:1 upon addition of glutamate, assuming that all glutamate was converted to NH_4^+. If anammox bacteria would use only the amine group to drive the anammox reaction there should be residual nitrite left. Absence of nitrite from the reactor together with ammonium accumulation suggested that denitrifying microorganisms had a competitive advantage over the anammox bacteria under the used growth conditions. Three batch additions of nitrite (day 36) in combination with ^{15}N-glutamate addition to the reactor resulted in an increase in ammonium concentrations during the first 30 min followed by the gradual consumption of the residual ammonium and the increase in nitrate concentrations upon nitrite addition (Fig. 5). This observation indicated that the anammox bacteria were limited by nitrite, but also at least part of the denitrifying community appeared to reduce nitrite very rapidly. Using labeled ^{15}N-glutamate revealed that around 5% of the amine group ended up in the N_2 pool which would make the total stoichiometric consumption 2 mol of NO_2^- per 1 mol of NH_4^+ during the course of this experiment, indicating that around 40% of nitrite is consumed by denitrifying microorganisms. In the further course of reactor operation the nitrite concentration stayed below the detection limit (5 μM), whereas nitrate concentrations seemed to stabilize and ammonium concentration gradually increased (Fig. 4). The amount of nitrite consumed per mol of NH_4^+ increased steadily from 1:1 on day 27 to almost 1:2 until day 61

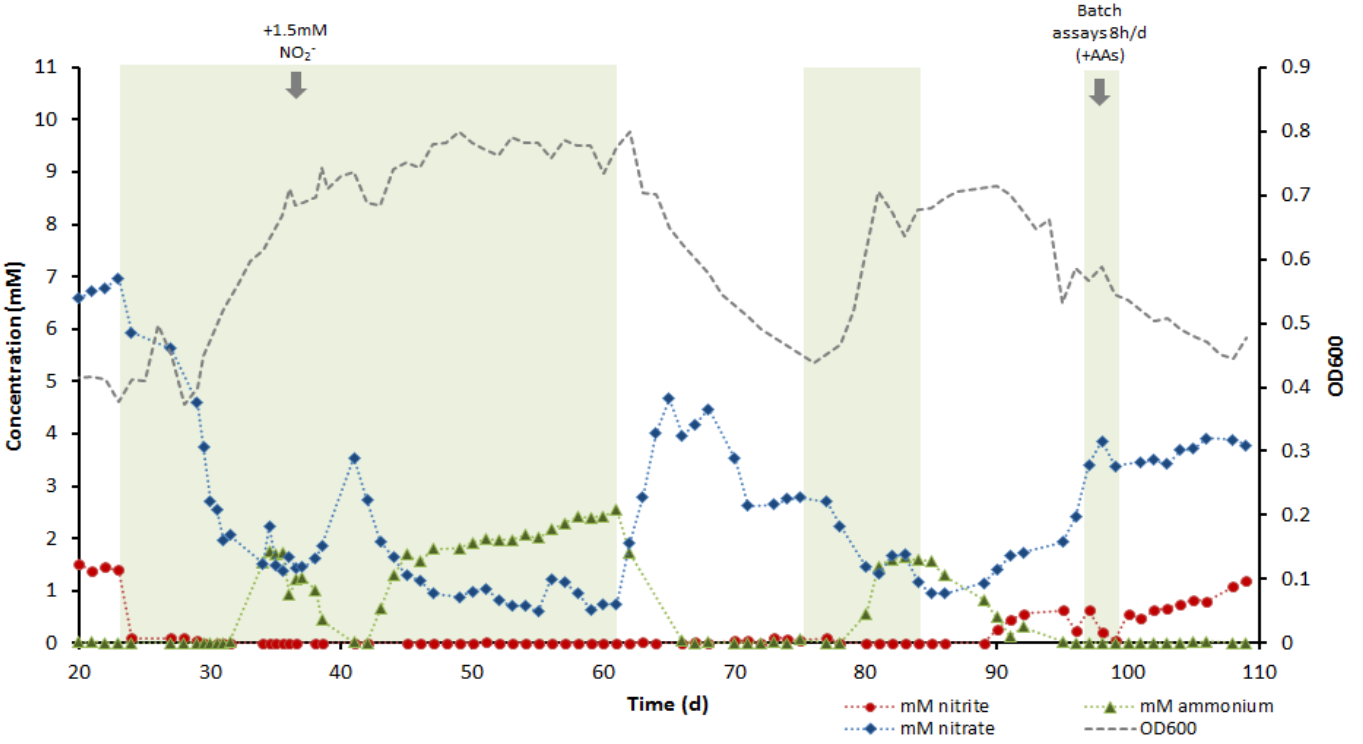


Figure 4: Nutrient concentrations and optical density (OD_{600}) of an ammonium-limited reactor system. Continuous feeding periods with glutamate are indicated as green areas. Arrows indicate experiments described in Fig. 5 and Fig.6).

again indicating the simultaneous occurrence of denitrification of anammox. At day 61 the amino acid addition was stopped to observe the development of the anammox and denitrifier competition in the absence of an organic electron donor. This resulted in the consumption of the accumulated ammonium and an increasing nitrate concentration indicating that the anammox reaction became the dominant nitrogen removal pathway. Consequently nitrite and ammonium in the reactor was converted with a stoichiometry 1:1.3 identical to previously reported values (Strous et al., 1999). Furthermore, stopping glutamate addition resulted in the washout of biomass from the reactor and the cell density (OD_{600}) in the reactor was almost halved within two weeks (Fig. 4) even though anammox bacteria were converting 5.4 mM NH_4^+ per day. These results indicated that the denitrifying community was washed out of the reactor. After the reactor reached steady state conditions (day 75), glutamate was continuously supplied to the reactor for a second time. The reactor responded to this second round of glutamate addition identically as the first time: the glutamate concentration was below detection limit, nitrate and nitrite concentrations decreased whereas ammonium concentration and cell density increased. If stable growth conditions for anammox bacteria were assumed, the other organisms converting amino acids in the system had a doubling time of approximately 8 days. As previously, stopping the addition of amino acids immediately restored the ammonium-limiting conditions and nitrate production, indicating anammox bacteria were again the dominant nitrite-reducing microorganisms in the system. Also the amount of nitrite consumed per 1 mol of ammonium dropped steadily to 1.18 mol.

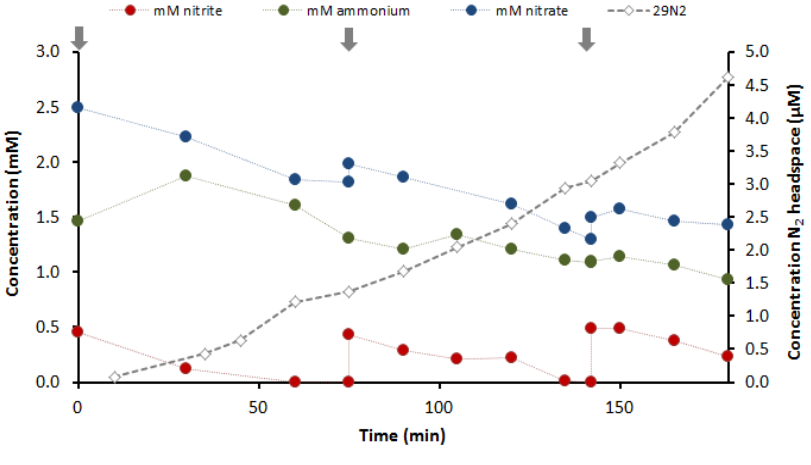


Figure 5: Monitoring of nutrient concentrations and $^{29}N_2$ production from ^{15}N-glutamate at day 36 upon the repetitive addition of 0.5 mM NO_2^- indicated by arrows.

Batch experiments with the amino acid adapted reactor

To study the turnover of nutrients under more controlled conditions, batch experiments using a reactor that was adapted to amino acid consumption were performed (day 97-99, Fig. 4). The headspace of the reactor was sealed, the influent pump supplying nitrite and ammonium was switched off and only ^{15}N-glutamate was supplied for several hours (Fig. 6, green areas). The biomass had not been supplied with amino acids for 12 days and was operated under ammonium limitation with stable nitrate and nitrite concentrations (Fig. 6 A). On day 1 glutamate addition was started 3.5 h after the reactor was sealed. Oxidation of ^{15}N-glutamate resulted in a steady increase of $^{29}N_2$, which was a direct indication that anammox bacteria used the released ^{15}N labeled amine group together with unlabeled nitrite. When the addition of glutamate was continued on day 2 and day 3, the residual nitrite was rapidly consumed and also the nitrate concentration dropped to 1.8 μM (Fig. 6B & 6C).

Also in this experiment nitrite limitation coincided with the accumulation of ammonium, which was produced with a rate of 14 $\mu M \cdot h^{-1}$ on day 2 increasing to 22 $\mu M \cdot h^{-1}$ on day 3. Identical to previous observations, ammonium oxidation resumed as soon as glutamate addition was stopped. Since nitrite was not added during this experiment, part of the consumed ammonium was dependent on nitrite production from nitrate reduction. Additionally, $^{28}N_2$ production was affected by stopping glutamate addition, indicating the dependence of denitrification on glutamate as electron donor. $^{29}N_2$ production decreased only by about 10% when glutamate addition was stopped (from 0.45 $\mu M \cdot h^{-1}$ to 0.40 $\mu M \cdot h^{-1}$). Again as previously observed, the nitrate concentration in the reactor was directly linked to glutamate addition: Nitrate was consumed when glutamate was supplied, but was produced again as soon as glutamate addition was ceased. This indicated that the rate of nitrate reduction coupled to amino acid oxidation was higher than the nitrate production rate of the anammox bacteria. Simultaneously an ammonium-limited *K. stuttgartiensis* enrichment that had not been previously exposed to amino acids was fed with glutamate using an identical setup. Figure 7 shows that glutamate uptake rates of a non-adapted culture is lower than the rate at which it is supplied, leading to its accumulation in the reactor system. At day 1 the anammox enrichment culture consumed 24 % of the glutamate supplied, increasing to 60 % on day 2 and 46 % on day 3 (Fig. 7). The addition of glutamate led to the production of $^{29}N_2$ from ^{15}N-glutamate (0.13 $\mu M \cdot h^{-1}$) and the consumption of nitrite (18 $\mu M \cdot h^{-1}$) (Supplementary Figure 1) at low rates without a lag phase, indicating that anammox bacteria might be

able to use the amino acids directly.

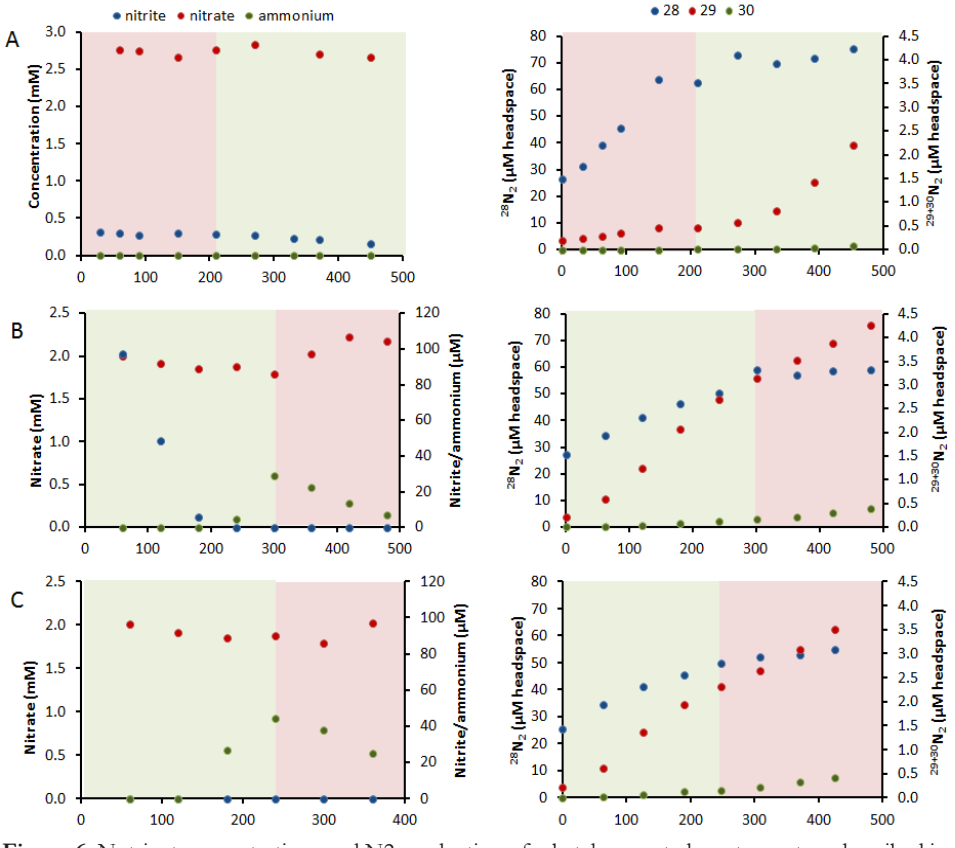


Figure 6: Nutrient concentrations and N2 production of a batch-operated reactor system described in Fig.4 that had been pre-exposed to amino acids for 3 consecutive days: A day 97, B day 98, C day 99. Batch operation was only maintained during the course of the experiment. The culture was monitored during continuous feeding of 10.5 mM 15N-glutamate (100 ml·min-1) (green area) and after the amino acid pump had been switched off (red area).

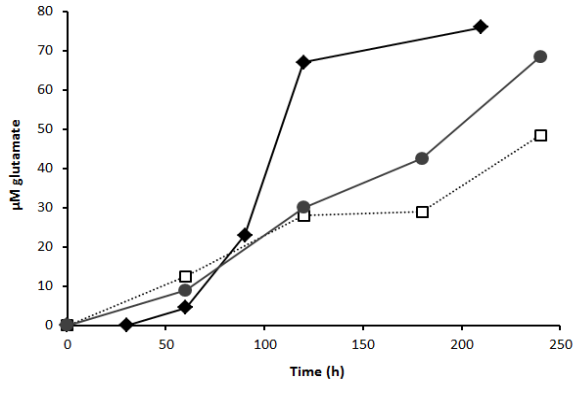


Figure 7: Glutamate accumulation in the reactor of an ammonium-limited, batch-operated reactor system not previously exposed to amino acids for 3 consecutive days. Batch operation was only maintained during the course of the experiment. The culture was monitored during 6-8h of continuous feeding of 10.5 mM ^{15}N-glutamate (100 ml·min^{-1}).

Community composition by metagenomics and FISH

To observe the effect of glutamate addition to the *K. stuttgartiensis* enrichment culture, the community composition of the reactor system was monitored with FISH and metagenomic analyses. The culture was examined under the microscope after 12 days of amino acid pulse-feeding and 49 days of continuous feeding respectively using fluorescent probes. Using AMX820 for anammox-specific detection and EUBmix as a general bacterial probe, showed an increase in the number, rod-shaped bacteria (Fig. 8).

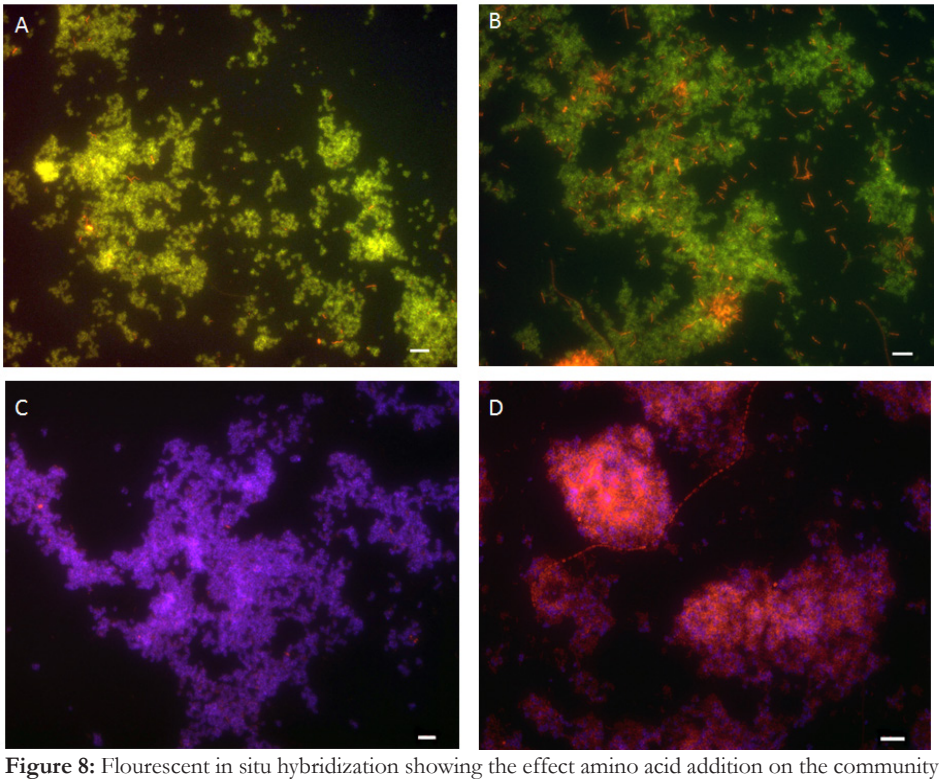


Figure 8: Flourescent in situ hybridization showing the effect amino acid addition on the community in the pulse-fed (A day 0, B day 12; AMX820 FLUOS (green) & EUBmix (red)) and continuous culture (C day 0, D day 49; AMX820 CY5 (blue) & EUBmix (red)). The scale bar represents 5 μM.

As an additional tool to study the microbial community of the reactor system we used metagenomic analyses. The pulse-fed (day 21) and continuous (day 58) systems were sampled when both systems were operating under steady state condition. In the continuously fed system 43% of all reads could be assigned to *Kuenenia stuttgartiensis* (average coverage 33-37%) after trimming whereas it was 58.3% of all reads in the pulse-fed system (average coverage 124-132%). Assembly of all acquired reads of the pulse-fed condition followed by annotation by MG-RAST (Meyer et al., 2008)

revealed *Bacteroidetes* (23.7%) and *Betaproteobacteria* (20.4%) to be the dominant other organisms in the community, followed by *Alphaproteobacteria* (11.1%), *Gammaproteobacteria* (9.4%), *Chloroflexi* (6.3%) and *Firmicutes* (6.9%). The sample from the reactor which was continuously fed with glutamate was also contained *Betaproteobacteria* (29%). *Alphaproteobacteria* (23.6%), *Bacteroidetes* (19.8%), *Gammaproteobacteria* (8.3%), *Firmicutes* (3.2%) and *Chloroflexi* (0.6%) as other dominant community members. Combing reads of both samples for a de novo assembly and then mapping back the reads of each sample separately against the assembled contigs resulted in a differential average coverage for pulse-fed and continuously fed conditions. Plotting these parameters against each other, including the GC% of each contig resulted in 7 bins (Fig. 9). Sequences were selected and blasted against a local database of 1442 representative genomes (Strous et al., 2012) to categorize the bins before submitting them to MG-RAST for annotation (Meyer et al., 2008). The high coverage bins (5,6 and 7) contained the majority of the reads and could be attributed to *K. stuttgartiensis*. The scattering of the anammox genome into variable bins could possibly be due to the differential enrichment of different *K. stuttgartiensis* strains. Bin 1 could be easily separated from the rest due to the high GC% contigs and high coverage. It contained mostly *Betaproteobacteria* with the *Rhodocyclales* (58%) and the *Burkholderiales* (24%) being the dominant orders. The remaining contigs were unclassified *Betaproteobacteria* (18%), possibly *Accumulibacter*-like organisms, or a minority of *Alpha-* and *Gammaproteobacteria*. *Betaproteobacteria* of both the *Rhodocyclales* and *Burkholderiales* orders often dominate man-made and natural systems with nitrate-reducing conditions in the presence of different substrates (Thomsen et al., 2007; Hesselsoe et al., 2009; Ishii et al., 2009). This might indicate that they play a role in amino acid oxidation using nitrate and/nitrite as terminal electron acceptor in our system. The similar average coverage of contigs grouped in bins 2 and 3 causes them to form a cluster (Fig. 9), that could only be separated based on differences in GC content of the contigs: Whereas sequences in bin 2 were low in GC (35±5%), bin 3 contained high GC contigs (62±1%). Also bin 3 contained mostly *Betaproteobacteria* of the order *Rhodocyclales* (16%) and *Burkolderiales* (12%), but also *Alphaproteobacteria* of the *Rhizobiales* order (15%), as well as *Chloroflexi* (10%) and *Firmicutes* (6%). The *Rhizobiales* are a diverse group of which several representatives are capable of heterotrophic denitrification under microaerophilic or anoxic conditions (Freitag et al., 1987; Delgado et al., 2003). Also the *Alphaproteobacteria*, might therefore contribute to denitrification.

Contigs in bin 3 and bin 4 belonged to at least two different types of *Bacteroidetes*

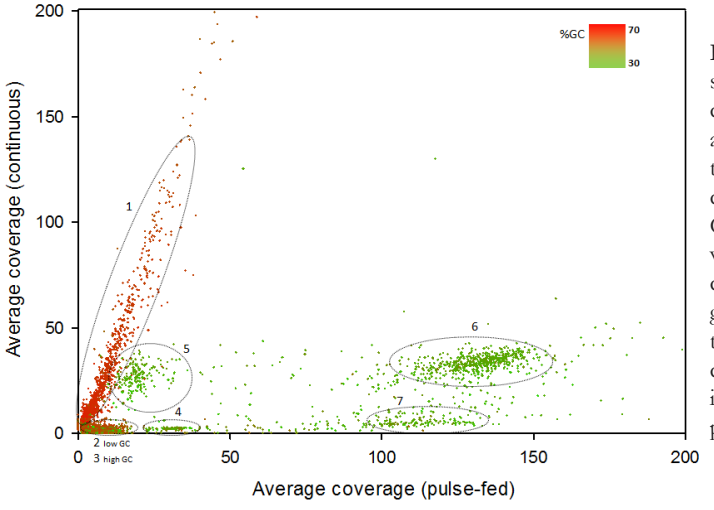


Figure 9: Scatterplot showing average coverage of de novo assembled reads from the pulse-fed and the continously-fed datasets. Only contigs ≥ 500bp were included. The colour gradient from green to red represents the GC content of contigs. The number indicate the bins with the presumable key players.

of the *Cytophaga–Flavobacteria* group with a slightly different coverage and GC content of the contigs. The sequences in both bins share only low similarity to cultured species and annotation resulted in high numbers of poorly characterized proteins. Members of the *Cytophaga-Flavobacterium* phylogenetic cluster have been described previously to play an important role in the decomposition of complex organic matter in anaerobic, marine habitats (Rosselló-Mora et al., 1999; Kirchman, 2002). They could fulfill a similar role in our reactor system, living off the fermentation of amino acids rather than of higher molecular weight substrates. After the annotation of all contigs, the denitrifying potential of each bin was analyzed based on the presence of the key genes involved in the process (Table 1). Almost all members of side population possessed one or more genes involved in the denitrification pathway. The complete denitrification pathway could only be found in the dominant *Rhodocyclales* and also in the *Bacteroidetes* species. Members of the *Cytophaga-Flavobacterium* phylogenetic cluster have been isolated under denitrifying conditions before (Heylen et al., 2006), but were more often associated with fermentation.

Conclusions

In the present study we showed that *K. stuttgartiensis* could use ammonium that was released during the oxidation and/or fermentation of amino acids under ammonium-limiting conditions and that cooperation between heterotrophic microorganisms and anammox bacteria could be possible under the described conditions despite a competition for nitrite. However, we were unable to demonstrate whether anammox bacteria themselves could convert amino

Table 1: Key genes involved in denitrification extracted from the different bins of the two amino acid-fed reactor system.

Gene	Bin	Order	Closest hit (blastx)	% Similarity (protein)
narG	1	*Burkholderiales*	*Achromobacter xylosoxidans*	90%
	2	unclassified Bacteria	*Candidatus* 'Omnitrophus fodinae'	82% 74%
	2	*Flavobacteriales*	*Arenibacter algicola*	68%
	3	*Rhizobiales*	*Afipia* sp. 1NLS2	81%
	6	*Candidatus Brocadiales*	*Candidatus* 'Kuenenia stuttgartiensis'	100%
napA	1	*Rhodocyclales*	*Azospira oryzae*	83%
	2	unclassified Bacteria	*Calditrix abyssi*	68% 72%
	3	*Rhodocyclales*	*Thauera* sp.	86%
nirS	1	unclassified β	*Candidatus* 'Accumulibacter sp. SK-11'	86%
	1	*Rhodocyclales*	*Sulfuritalea hydrogenivorans* sk43H	76%
	3	Anaerolineales	*Anaerolinea thermophila*	77%
	3	*Burkholderiales*	Burkholderiaceae bacterium N52	82%
	6	*Candidatus Brocadiales*	*Candidatus* 'Kuenenia stuttgartiensis'	100%
nirK	2	*Flavobacteriales*	*Chryseobacterium hispalense*	78%
	4	*Sphingobacteriales*	*Pedobacter arcticus*	63%
norB	1	*Rhodocyclales*	*Azospira oryzae*	80%
	2	*Flavobacteriales*	*Formosa* sp. AK20	54%
	4	*Sphingobacteriales*	*Flavihumibacter solisilvae*	86%
nosZ	1	*Rhodocyclales*	*Azospira oryzae*	92%
	2	*Flavobacteriales*	*Capnocytophaga cynodegmi*	78%
	2	*Flavobacteriales*	*Flavobacterium columnare*	74%
	2	*Sphingobacteriales*	*Pedobacter saltans*	74%
	3	*Burkholderiales*	*Acidovorax* sp. MR-S7	86%
	3	*Flavobacteriales*	*Imtechella halotolerans*	65%

acids. Pulse-feeding and continuous addition of amino acids at concentrations used in this study facilitated the growth of denitrifying microorganisms. The resulting constant production and consumption of nitrate, ammonium and nitrite mimicked how different clades of microorganisms would interact in nature and enabled us to study such interactions, which could also take place in environments with similar conditions i.e. marine oxygen minimum zones or other nitrate-rich ecosystems to our model reactor system, in molecular detail. The results reported here provide the first insight into how denitrifiers and anammox bacteria interact in a system with amino acids as the only organic electron donor and lay the foundation for the study of these interactions in nature.

Experimental procedures

Next generation sequencing

DNA was extracted from 8 ml co-culture biomass using the PowerSoil® DNA isolation kit (MO BIO, USA) following the protocol. Concentrations were determined spectrophotometrically using the NanoDrop (Thermo Scientific). 100 ng genomic DNA was sheared with sonnication using the Bioruptor® Standard (Diagenode), with 11 cycles of 30 sec sonication and 90 sec off and 6 additional cycles of 60 sec sonication and off. Further library

preparation was performed using the Ion Plus Fragment Library Kit (Life technologies, USA) following manufacturer's instructions. Size selection of the library was performed using an E-gel 2% agarose gel (Life technologies, USA) resulting in a mean fragment length of 472 (19-5-2014) and 435 bp (21-08-2014). Emulsion PCR was done using the Onetouch 200bp kit and sequencing was performed on an IonTorrent PGM using the Ion PGM 400bp sequencing kit and an Ion 318 chip (Life technologies, USA), resulting in 3.798.027 (19-5) and 1.319.179 (21-8) reads of average length 262 bp.
Analysis of reads was performed using CLC genomics workbench 7.5.2 (CLCbio, Denmark). The reads were length and quality score trimmed (>100bp, 0.05) resulting in a dataset of 3.416.500 and 1.209.550 reads respectively. A de novo assembly included in the program mapping the reads back to the contigs (Length fraction 0.5, similarity fraction 0.8) was performed using the default settings. This resulted in 55.607 and 12.155 contigs. Bins were selected according to average coverage and GC% and submitted to the RAST annotation server (Aziz et al., 2008).

Fluorescence In Situ Hybridization
Biomass was fixed in a 4% w/v paraformaldehyde solution for 1-3h on ice. After washing cells were stored at -20 ºC in a 50% v/v 1 X PBS and 50% v/v ethanol solution. Probes included EUBmix (Daims et al., 1999) and AMX820 (Schmid et al., 2001). Staining with DAPI was included as a positive control.

Analytical methods
Nitrite was measured colorimetically at 540 nm after a 15 min reaction of 1 ml sample (0.1 – 0.5 mM nitrite) with 1 ml 1% sulfanilic acid in 1 M HCl and 1 ml 0.1% naphtylethylene diaminedihydrochloride. Ammonium was measured at 420 nm on a Cary Eclipse Fluorescence Spectrophotometer after reaction with 10% ortho-phthaldialdehyde as has been described previously (Taylor *et al.*, 1974). Nitrate was measured by converting nitrite and nitrate in the samples to nitric oxide in a saturated solution of VCl_3 in 1M HCl at 95 °C. NO was then measured by the Nitric Oxide Analyzer NOA280i (GE Analytical Instruments, USA) according to the protocol. Amino acids were measured in a Varian 920-LC (Agilent Technologies) by injecting 10 µl sample into an in the ProStar 420 autosampler for derivatization with FMOC-Cl. Separation was done on a Varian Pursuit XR Ultra C18 column in a high Pressure Liquid Chromatography (HPLC) system using 30% v/v acetonitrile and 7% v/v tetrahydrofuran in milliQ containing 5.6mM trisodiumcitrate dehydrate and 3.8 mM tetramethylammonium chloride as mobile phase. Elution was done with 1.1 $ml \cdot min^{-1}$ at 36 °C within 35 min. The UV absorbance spectrum of

was measured at 269 nm and the fluorescence detection at 265/340 nm. 250 pmol norvaline was added to each sample as internal standard.

Reactor operation

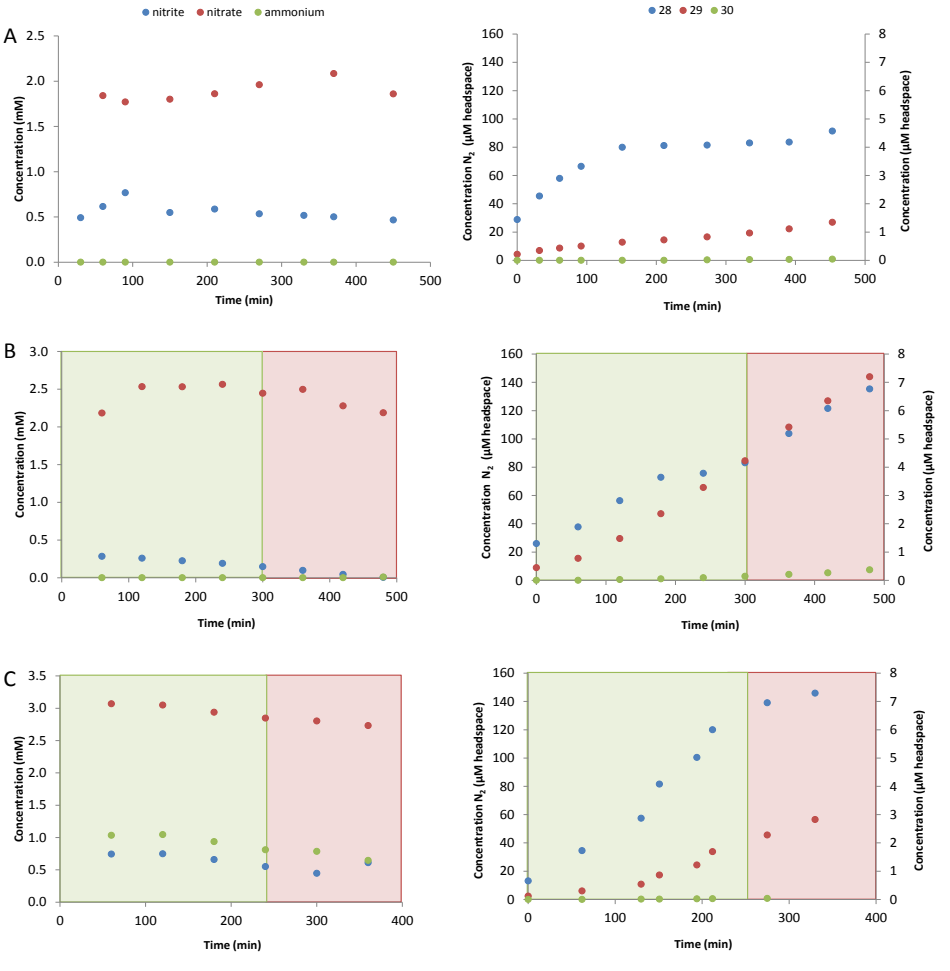
A membrane reactor (working volume 1.5 l) was used to grow *Kuenenia stuttgartiensis* single cells. Medium generally used to cultivate anammox bacteria (van de Graaf et al., 1996) was added to the reactor with a flow of 450 $ml \cdot d^{-1}$. The pH was controlled at 7.4 with a sterile 100 $g \cdot l^{-1}$ $KHCO_3$ solution and. The reactor was temperature controlled at 30°C and the stirring speed was set to 125 rpm. The reactor was operated under anaerobic conditions by constant flushing with Arg/CO_2 (10 $ml \cdot min^{-1}$). Ammonium was limiting with influent nitrite and ammonium concentrations of 25.9 mM NO_2^- and 18.5 mM NH_4^+ during stable operation. Sodium glutamate, serine and glycine were added by pulse feeding as 630 μM directly to the reactor or sodium glutamate only was added continuously from a sterile 10.5 mM stock with a flow of 100 $ml \cdot d^{-1}$.

Activity assays

Whole reactor activity assays were performed with labeled amino acids (^{15}N-glutamate or ^{15}N-glycine). The avoid washout of labeled gas compounds the reactor was operated as batch during 8 h of measurement. The production of $^{28}N_2$, $^{29}N_2$ and $^{30}N_2$ was followed by gas chromatography coupled to mass spectrometry on an Agilent 6890/5975c MSD. For the quantification of N_2 standard curves a commercially available gas mixtures was used containing 1% N_2. Nitrate, ammonium, nitrite and amino acids were determined as described earlier.

Acknowledgements

We would like to thank Paul van der Ven for amino acid analysis. The work was funded by ERC AG (232937) to M.S.M. Jetten & L. Russ, by NWO VENI grant (863.11.003) to B. Kartal and by the NWO SIAM Gravitation grant (24002002).



Supplementary Figure 1: Nutrient concentrations and N_2 production of a batch-operated reactor system that had not been pre-exposed to amino acids for 3 consecutive days: **A** day 1, **B** day 2, **C** day 3. Batch operation was only maintained during the course of the experiment. The culture was monitored during continuous feeding of 10.5 mM ^{15}N-glutamate (100 ml·min^{-1}) (green area) and after the amino acid pump had been switched off (red area).

Chapter 5

Chapter 6

Genome analysis and heterologous expression of acetate-activating enzymes in the anammox bacterium *Kuenenia stuttgartiensis*

Russ, L., Harhangi, H.R., Schellekens, J., Verdellen, B., Kartal, B., Op den Camp, H.J.M., and Jetten, M.S.M. (2012) Genome analysis and heterologous expression of acetate-activating enzymes in the anammox bacterium *Kuenenia stuttgartiensis*. *Arch Microbiol* **194:** 943-948.

Summary

Anaerobic ammonium-oxidizing bacteria were recently shown to use short-chain organic acids as additional energy source. The AMP-forming acetyl-CoA synthetase gene (*acs*) *of Kuenenia stuttgartiensis*, encoding an important enzyme involved in the conversion of these organic acids, was identified and heterologously expressed in *Escherichia coli* to investigate the activation of several substrates, that is, acetate, propionate and butyrate. The heterologously expressed ACS enzyme could complement an *E. coli* triple mutant deficient in all pathways of acetate activation. Activity was observed toward several shortchain organic acids, but was highest with acetate. These properties are in line with a mixotrophic growth of anammox bacteria. In addition to acs, the genome of *K. stuttgartiensis* contained the essential genes of an acetyl-CoA synthase/CO dehydrogenase complex and genes putatively encoding two isoenzymes of archaeal-like ADP-forming acetyl-CoA synthetase underlining the importance of acetyl-CoA as intermediate in the carbon assimilation metabolism of anammox bacteria.

Introduction

Bacteria capable of anaerobic ammonium oxidation (anammox) derive their energy for growth from the conversion of ammonium and nitrite into dinitrogen gas, thereby constituting a significant sink for fixed nitrogen under anoxic conditions (Arrigo 2005; Lam and Kuypers, 2011). Cellular carbon is hypothesized to be fixed via the acetyl-coenzyme A (CoA) pathway, suggesting a chemolithoautotrophic lifestyle (Schouten et al. 2004; Strous et al. 2006). throughout this pathway, organic carbon is formed by reducing CO_2 to CO and subsequently to cellular components via acetyl-CoA. Interestingly, it was shown recently that anammox bacteria have a more versatile metabolism than previously assumed: several genera were able to oxidize organic compounds to CO_2 with nitrate and/or nitrite as electron acceptor, possibly refixing the CO_2 via the acetyl-CoA pathway and fueling the catabolic reaction with nitrite (Guven et al. 2005; Kartal et al. 2007b, 2008). Although the nitrate reduction pathway has been elucidated (Kartal et al. 2007a), the underlying biochemical pathway for organic acid oxidation is still unknown. The abundance of genes potentially involved in organic acid conversion points to its importance in anammox metabolism. The metabolism of acetate is commonly initiated by its activation to acetyl-CoA that is an essential intermediate of various anabolic and catabolic pathways and has a central role in the carbon metabolism in all three domains of life (Wolfe 2005; Ingram-Smith et al. 2006a). At least five different ways to synthesize acetyl-CoA are known at present (AMP-forming acetyl-CoA synthetase (ACS), ADP-forming acetyl-CoA synthetase (ACD), acetate kinase/phosphotransacetylase (ACKA and PTA), CO dehydrogenase/acetyl-CoA synthase (CODH/ACS) and acetate-CoA transferase). One of these enzyme complexes, an AMP-forming acetyl-CoAsynthetase, is essential for the synthesis and conversion of acetate to acetyl-CoA and was experimentally investigated in this study. ACS catalyzes the direct formation of acetyl-CoA from acetate, ATP and CoA and is present in nearly all organisms. In prokaryotes, it is known to operate often in an assimilatory route during growth on low acetate concentrations (≤10mM) (Wolfe 2005). It is a member of a family of AMP-forming enzymes that catalyze two-step reactions in which an acyl-adenylate intermediate is formed and pyrophosphate is released (Starai and Escalante-Semerena 2004). The analysis of the genome sequence of the anammox bacterium *Kuenenia stuttgartiensis* revealed several open reading frames (ORFs) with similarity >30% to known acetate-activating enzymes. Their presence gave a first indication about the route of acetate utilization in anammox bacteria, although the incorporation of acetate-derived carbon into cellular biomass

could not be detected so far (Kartal et al. 2007b, 2008). The present study focused on the functional expression of a putative AMP-forming ACS, the most abundant acetate-activating enzyme in the proteome of *K. stuttgartiensis* encoded in ORF kustc1128 (Kartal et al. 2011). An *ackA-pta-acs* triple mutant of *E. coli* was complemented with the *K. stuttgartiensis acs* gene resulting in recovery of growth on acetate. To investigate the substrate specificity and kinetic properties of the putative ACS, the *acs* gene was overexpressed in the host *E. coli* RosettaTM 2. The potential physiological role of acetate conversion in vivo was determined by colorimetric determination of acetyl-CoA formed from acetate by *K. stuttgartiensis*. This is the first time that an anammox enzyme could be functionally expressed in a heterologous host and that its properties could support an important role in the carbon assimilation metabolism of *K. stuttgartiensis*.

Results and discussion

Phylogeny and expression of genes encoding acetate-activating enzymes in *K. stuttgartiensis*. A thorough analysis of the *K. stuttgartiensis* genome assembly revealed seven protein-coding ORFs (>30 % identity) that were possibly involved in acetate metabolism as well as key enzymes of the acetyl-CoA pathway (Supplementary Table S1). Such a redundant repertoire of acetate- and acetyl-CoA-converting enzymes has not been observed in chemolithotrophic bacteria before. The most highly expressed ORF associated with acetate conversion in *K. stuttgartiensis* was kustc1128 encoding a putative AMP-forming acetyl-CoA synthetase (589 aa, calculated molecular mass 67 kDa; Table 1).

Table 1 Relative gene expression and coverage in the proteome of potential acetate-activating enzymes in *K. stuttgartiensis*

Locus	Annotation	Gene expression[a]	Peptides[b]
kusta0048	acetate-CoA ligase (ADP-forming); β-domain (*acdB*)	0.70	0
kustb2015	acetate-CoA synthetase/acetate-CoA ligase	0.63	0
kustc0502	acetate-CoA ligase (ADP-forming); α-domain (*acdA*)	0.46	0
kustc1128	acetyl CoA synthetase (*acsA*)	1.40	6 (13%)
kustee3169	acetyl CoA synthetase (ADP-forming)	0.64	1 (2%)
kuste3170	hypothetical phsophotransacetylase protein	0.50	0
kuste3344	phenylacetate-CoA-ligase (*paaK*)	0.31	0

[a] Relative expression: (#reads x read length/ORF length, relative to overall coverage
[b] # of peptide hits (percentage coverage)

Using the *K. stuttgartiensis* kustc1128 sequence as a template, a similar gene could also be identified in several other available anammox metagenomes

(Gori et al. 2011; Harhangi et al. 2012; van de Vossenberg et al. 2012) suggesting a central role in anammox bacteria. The identities among the anammox ACS were >60 %. Phylogenetically, kustc1128 and other anammox homologues clustered at maximum identities of 57 % with representatives of the Archaea and Firmicutes (Fig. 1). These and several proteo- and actinobacterial sequences were affiliated to a larger cluster of acetyl-CoA synthetases that share a distinct domain structure (cluster I). The amino acid sequence differs significantly (<40 % identity) from the commonly described ACS (cluster II). A homologue of this cluster II could not be identified in *K. stuttgartiensis* after extensive analysis that makes kustc1128 the only ACS-like encoding gene.

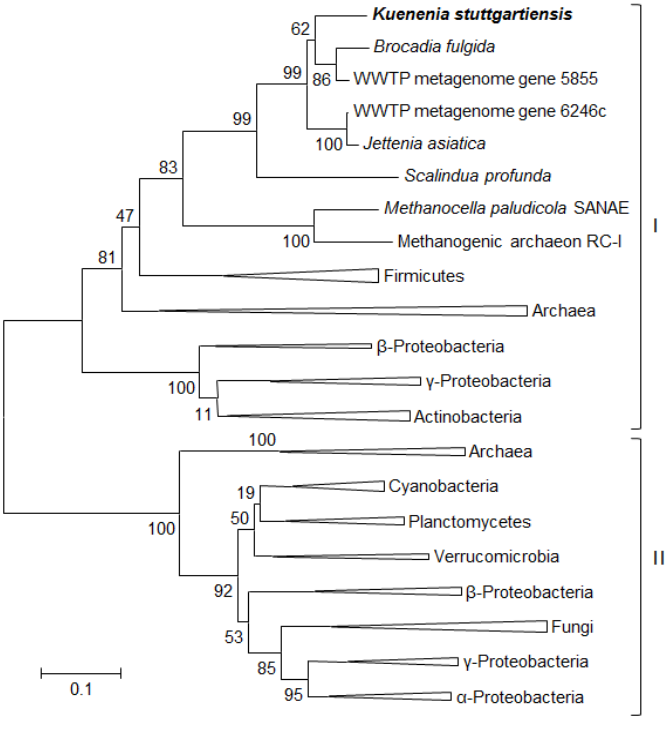


Figure 1: Neighbor-joining tree of phylogeny estimated by ClustalW included in the MEGA 5.05 software package, showing acetyl-CoA synthetase (AMP-forming) homologues with two different conserved domain architectures: cluster I and cluster II. Values at the internal nodes indicate bootstrap values based on 1000 iterations.

Closest hits were obtained (>60 %) to the ACS of hydrogenotrophic methanogens of the rice cluster I (RC-1), which use the enzyme for acetate assimilation (Erkel et al. 2006). Like the RC-1 archaeon, *K. stuttgartiensis* also encodes a putative vacuolar-type H^+-translocating inorganic pyrophosphatase (kustd1836) that might function as a transmembrane proton pump (Serrano et al. 2004, 2007; Bielen et al. 2010). The pyrophosphate (PPi) released as a by-product of acetate activation, could theoretically be used to establish a proton motive force and thereby recover a quantity of the energy previously

invested (Jetten et al.1992).

Mutant complementation tests

An *E. coli* strain (AJW807) deficient in all three pathways of acetate activation was used to determine the functionality of kustc1128 as an AMP-forming ACS.

The pET30a-containing mutant expressing kustc1128 upon induction with IPTG was able to grow on acetate as carbon source. Protein was isolated from a LB-grown *E. coli* AJW807 control group and from three different complemented mutants, grown on M63 supplemented with acetate. In the complemented mutant clones, the ACS enzyme had a specific activity of 57 nmol min^{-1}·mg $protein^{-1}$. This is in concert with earlier described ACS activity tests of the *ack* mutant of *E. coli* K12 (Brown et al. 1977). The mutant incapable of acetate activation showed significantly less activity (0.8 nmol min^{-1} mg $protein^{-1}$). This remaining activity could be due to the background activity of phenylacetate-CoA ligase (Brunner et al. 1975) or long-chain acyl-CoA ligase (Kornberg and Pricer 1953). The expression of ORF kustc1128 restored the acetate-activating capacities in an *E. coli ackA-pta-acs* triple mutant, indicating its physiological role as an active AMP-forming ACS in *K. stuttgartiensis*.

Substrate specificity and kinetic parameters

The heterologous expression as a His-tagged protein allowed rapid purification of the *K. stuttgartiensis* ACS over a Ni–NTA column. The His-tagged ACS was loaded on 10 % SDS-PAGE (Supplementary Fig. S1). Only one prominent band was visible at the expected mass. MALDI-TOF MS analysis of the purified ACS after a trypsin digestion confirmed its identity as kustc1128 (Supplementary Fig. S1; sequence coverage 35.8 %). The purified enzyme was tested for substrate specificity and kinetic properties. Activity toward acetate was the highest among tested organic acids (130 ± 9 nmol min^{-1} mg $protein^{-1}$; n = 6). The enzyme retained its activity over a wide pH range with

Organic acid	Rate (nmol·min^{-1}·mg $protein^{-1}$)	% of rate with acetate
Acetate	130.3	100
Propionate	114.9	88
Formate	84.8	65
Butyrate	40.5	31
Iso-butyrate	39.1	30

Table 2: Specific activity of the purified ACS-like enzyme with different organic acids.

an optimum around pH 7.
Activity decreased with only 20 % between pH 6.5 and 8.5 (Supplementary Fig. S2). Relative to acetate, the propionate and formate conversion rates were reduced to 84 and 66 %, respectively, whereas butyrate (31 %) and isobutyrate (34 %) were converted at even lower rates (Table 2). The K_m for acetate was estimated at 0.2 mM that is comparable with K_m values of *E. coli, Haloarcula marismortui* and *Azotobacter aceti* ACS (O'Sullivan and Ettlinger 1976; Kumari et al. 1995; Bräsen and Schönheit 2005a) and well within the range of other described ACS enzymes (0.003–1.2 mM, (Bräsen et al. 2005b; Li et al. 2012) (Fig. 2). It has been reported previously that ACS could convert other organic substrates, in particular propionate, albeit with a significantly lower specific activity (Jetten et al. 1989; de Cima et al. 2007; Ingram-Smith and Smith 2007). The *K. stuttgartiensis* enzyme shows only a slightly reduced activity with propionate compared to acetate indicating a broad substrate range. Also, the conversion of longer substrates such as isobutyrate and butyrate is not a common characteristic and has been shown only for the archaeal ACS enzymes in *Archaeoglobus fulgidus* (ACS2) and *Pyrobaculum aerophilum* (Bräsen et al. 2005; Ingram-Smith and Smith 2007). It is hypothesized that the broad substrate specificity is established by a substitution in one of the four conserved residues in the acetate-binding pocket that determines the specificity of the acyl-substrate (Ingram-Smith et al. 2006b). Based on sequence comparison, the Ile^{312} in the *K. stuttgartiensis* ACS is replaced by Val, a trait conserved among the described organisms sharing similar catalytic properties.

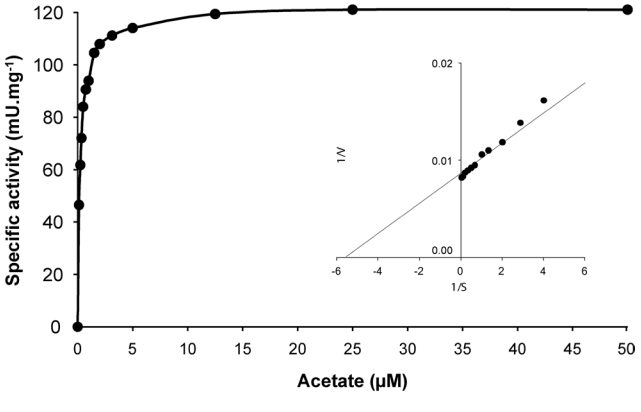


Figure 2: Rate dependence of the potential *K. stuttgartiensis* ACS activity at different acetate concentrations. The inset shows a plot of the reciprocal velocity against the reciprocal of the substrate concentration.

Acetate conversion in Kuenenia stuttgartiensis
That anammox bacteria can use organic acids as electron acceptor has been shown previously (Kartal et al. 2007b, 2008). The fate of those organics is until now still speculative, but all known pathways of acetate or propionate

conversion proceed via acetyl-CoA, which would also be the end product of carbon fixation in anammox bacteria. As incorporation of acetate-derived carbon has not be shown yet, whole cells of *K. stuttgartiensis* were incubated with acetate, ATP and HSCoA and the conversion into acetyl-CoA was determined by measuring the Fe^{3+}-acetyl hydroxamate complex formation. The rate of acetyl-CoA formation was significantly higher than for the complemented *E. coli* mutant (7.7 µmol min^{-1} mg^{-1}) (Fig. 3). The conversion of acetate to acetyl-CoA increased linearly with the amount of cells added, whereas boiled cells did not show any activity. Considering this assay relies on total protein concentrations, this rate could very well fit with that of the

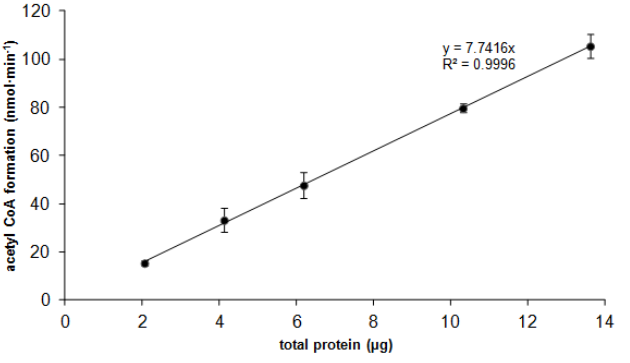


Figure 3: Formation of acetyl-CoA from potassium acetate in response to the addition of different amounts of *K. stuttgartiensis* whole cells.

heterologously expressed, His-tag purified enzyme.

In the present study, we could show that acetate could be activated by kustc1128, an *acs*-like protein, as well as whole cells of *K. stuttgartiensis* suggesting that indeed the reductive acetyl-CoA pathway was used by anammox bacteria as previously suggested. Such acetate activation could also lead to the direct incorporation of acetate into cell biomass by anammox bacteria.

Additionally, the PP_i released upon the formation of acetyl-CoA could be used to translocate protons by an H^+-translocating pyrophosphatases building up a proton motive force over the anammoxosomal membrane, which is central to the anammox catabolism (Kartal et al. 2011). Recently, it was shown that the ATP-consuming reaction of ACS could be coupled to ATP-producing processes, a possibility that gives interesting perspectives regarding further research on anammox carbon metabolism (Mayer et al. 2012).

Experimental procedures

Identification of putative acetate-activating enzymes

To identify gene orthologs of acetate-activating enzymes in the genome of *Kuenenia stuttgartiensis* (Strous et al. 2006), a local BlastX search (National

Center for Biotechnology Information, Bethesda) was performed using the *acs* sequence of *Escherichia coli*, *Methanosaeta concilii* and *Saccharomyces cerevisiae*, the *acdA* and *acdB* sequences of *Archaeoglobus fulgidus*, and *ack-pta* genes of *E. coli* and *Methanosarcina barkeri* as queries.

Preparation of DNA and construction of complementation vectors

Kuenenia stuttgartiensis biomass was enriched in a sequencing batch reactor (SBR) under conditions commonly used for anammox bacteria (Strous et al. 1998). Cells were harvested by centrifugation at 6,000 rpm for 10 min, resuspended in 20 mM HEPES buffer (pH 7) and disrupted in a French pressure cell. DNA was extracted as described before (Juretschko et al. 1998) and subsequently served as template for a Phusion-based high fidelity PCR (Finnzymes, Finland) with ORF kustc1128-specific primers extended by a BamHI and a HindIII restriction site, respectively (acs-F5'GGATCCATGAATAAGACTGAAATAATAAATAAAC-3' and acs-R5'AAGCTTATCTTCAAGTGTAGAAATATCTC-3'). Amplification was initiated with a denaturation step at 95 °C for 5 min and continued with an optimized amplification program of 30 cycles (1 min at 95 °C; 1 min at 52.5 °C; 3.5 min at 72 °C) with a final elongation step at 72 °C for 10 min. PCR products were cloned into the TopoTA vector (Invitrogen, UK) and transformed by heat shock into *E. coli* TOP10 (Invitrogen, UK) competent cells. Plasmids were isolated, digested with BamHI and HindIII and gel-purified with the Qiaex II Gel extraction kit (Qiagen Benelux, The Netherlands). The construct was ligated into the pET30a (Novagen, Germany) vector system.

Mutant complementation

The constructed pET30a-*acs* vector was used to transform the *ackA-pta-acs* triple mutant *E. coli* AJW807 (Kumari et al. 1995), by the heat shock method. Progenies were selected on minimal medium (M63) supplemented with 10 mM Na-acetate, 0.02 % w/v glucose and 100 μg/ml ampicillin after induction with 75 μM isopropyl-β-d-thiogalactopyranoside (IPTG). After 3 days of incubation at 37 °C, colonies were transferred to Luria–Bertani medium plus 100 μg/ml ampicillin. Using the FlexiPrep™ kit (GE Healthcare Benelux, Belgium), plasmids were extracted to confirm the sequence accuracy by the Sanger method. Three colonies were selected from plate and resuspended in 1× PBS $MgCl_2$ after washing. Cells were lysed by four sonification intervals of 15 s and used in the activity assay as described below.

Expression and purification of recombinant ACS-like enzymes

The recombinant plasmids were used to transform *E. coli* JM109. Plasmids were extracted from the overnight culture to confirm sequence accuracy by Sanger sequencing. Flawless constructs were transformed into the expression host *E. coli* Rosetta™ 2 (DE3) (Novagen, Germany). Cells were grown at 37 °C in Luria–Bertani medium supplemented with 100 µg/ml kanamycin and 34 µg/ml chloramphenicol to an OD_{600} of approximately 0.6, and then the expression was initiated by the addition of 1 mM IPTG. After incubation for 3 h at 30 °C (final OD_{600} 1.0–1.2), cells were harvested by centrifugation from a total culture volume of 1 L. The His-tagged protein was purified with the Ni–NTA Spin kit (Qiagen Benelux, The Netherlands) according to the protocol of protein purification under native conditions from *E. coli* lysates using buffer NTI-10 and an additional disruption step in the French press for cell lysis. After confirming that the purified enzyme was the gene product of kustc1128 by MALDI-TOF MS analysis (Bruker Biflex III; Bruker Daltonics, USA), the eluted fractions were used for activity assays (described below).

Preparation of K. stuttgartiensis whole cells

Cells were harvested by centrifugation (20 min, 4,000 rpm at 4 °C) and concentrated in 20 mM $NaHCO_3$ buffer (pH 7.4). Negative controls were prepared by boiling cells for 15 min. The cells were used in an activity assay as described below.

Enzyme assays and kinetic analysis

Assays were performed routinely at 37 °C in a total volume of 1 mL. The formation of acetyl-CoA from acetate, ATP and HSCoA was assessed by monitoring the Fe^{3+}-acetyl hydroxamate complex formation from acetyl-CoA and hydroxylamine at 540 nm as adapted from Berg (1956). The assay mixture contained 145 mM Tris/HCl (pH 7.5), 10 mM $MgCl_2$, 200 mM potassium acetate, 120 mM hydroxylamine hydrochloride (pH 7.5), 10 mM ATP, 0.47 mM HSCoA, 10 mM reduced glutathione and purified enzyme or whole cells. The reaction was stopped at 15 and 30 min, respectively, by adding 10 % TCA and 2.5 % $FeCl_3$. This assay was used to determine the specific activity and apparent K_m values of the acetyl-CoA synthetase (AMP-forming) in acetate-grown cells of *E. coli*, the ACS activity in the fractions of the His-tag-purified enzyme preparations and the acetate conversion by whole cells of *K. stuttgartiensis*.

Protein alignment and phylogenetic analysis

Sequences were aligned using the ClustalW multiple sequence alignment tool

based on the Gonnet matrix options included in the MEGA 5.05 software (Tamura et al. 2011). A phylogenetic tree was constructed using both the neighbor-joining and the maximum likelihood algorithms of MEGA 5.05. Bootstrap values at the internal nodes were calculated from 1,000 iterations.

Proteome and transcriptome

Materials and methods of proteome and transcriptome isolation and data analysis are described elsewhere (Kartal et al. 2011).

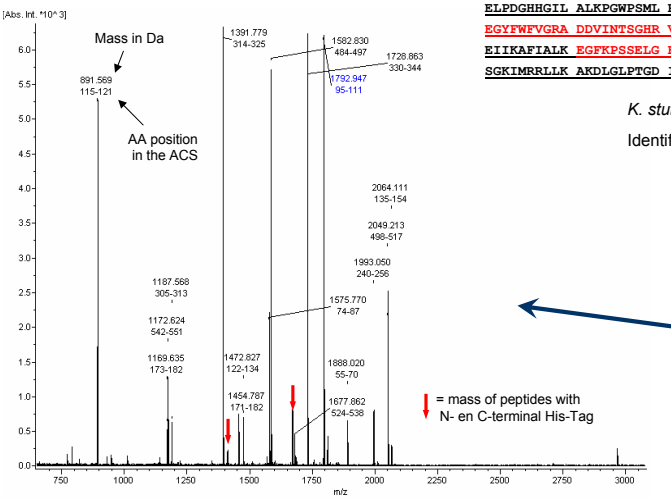
Acknowledgments

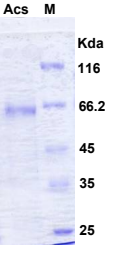
We thank A. J. Wolfe, Loyola University Chicago USA, for providing the triple mutant strain AJW807. This research was supported by grant 232937 from the European Research Council.

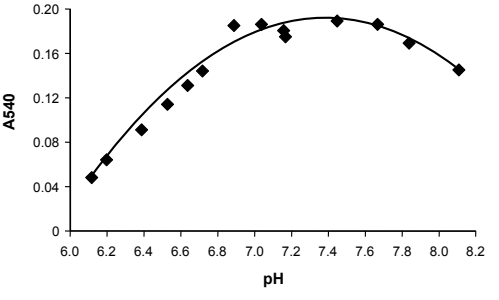
Supplementary Figure S1: The Ni-NTA purified acetyl CoA synthetase was separated on a 10% SDS PAGE (right bottom). The band at about 65 kDa was excised and trypsinised. The trypsin digest was analyzed by Maldi tof MS (spectrum below) to confirm the identity of the ACS. The ACS sequence with the identified parts is showed at the right part.

MHHHHHHSSG LVPRGSGMKE TAAAKFERQH MDSPDLGTDD DDKAMADIGS
MNKTEIINKH PEAFNLICYE DQHKNFSWET VKKELGVGGN KVNIAYEAID
KHATTWRKNK VALYWEGSDG THLKYTFQEL KILSDKCANM LQSLGVGKGD
RVFLFLPRLP ELFINMIAIA KLGAISGPMF SAFGPDAVRD RLQNSEAKVL
ITTPELKERV DAVLWELPKL ERIVLVSVKE DYELEEGNVC YKTLMKDAPE
RFEMEWMDME DPLYLLYTSG TTGKPKGITH VHNDMISYYI TTKWSLDLRD
DDIYWCTADP GWVTGMVYGM WGPWLNGVSM YIYDGRFDVN KWYEAIQSYK
ITVWYTAPTA LRMLMKSGDY LVAQYDLESL RYICSVGEPL NPEVIKWGMN
VYNLPIHDTW WQTETGSIMI ANYPCIPIKP GSMGKPFPGI KAAIIDSEGN
ELPDGHHGIL ALKPGWPSML RKVWGDEGRF NEYFNITGWY TTGDTAYKDE
EGYFWFVGRA DDVINTSGHR VGPFEVESAL LEHRAVAEAG VIGKPDPERG
EIIKAFIALK EGFKPSSELG EEIKKFIKHH LAAHAYPREI EFCENLPKTR
SGKIMRRLLK AKDLGLPTGD ISTLEDKLAA ALEHHHHHH

K. stuttgartiensis Acs (underlined)
Identified by Maldi-tof MS (in red)







Supplementary Fig. S2 Effect of pH on the activity of the heterologous expressed acetyl CoA synthetase of *K. stuttgartiensis* (kustc1128).

Supplementary Table S1 Description and characterization of *K. stuttgartiensis* ORFs possibly participating in acetate or acetyl-CoA interconverting reactions.

***K. stuttgartiensis* genome**				**Blast Output**		
Query	Annotation	Length	Domains	Organism	Annotation	E-value
kustb0215	acetyl-coenzyme A synthetase/acetate-CoA ligase (caiC)	528aa	COG0318	uncultured delta proteobacterium ADI17226.1	acyl-CoA synthetases (AMP-forming)/AMP-acid ligases II	5.00E-88
kustc0502	acetyl-CoA synthetase (ADP-forming); α- domain (acdA)	462aa	TIGR02717	*Methanosarcina mazei* Go1 NP_632382.1	acetyl-CoA synthetase, α-subunit	1.00E-101
kustd1545	CO dehydrogenase/acetyl-CoA synthase; α-subunit	727aa	PRK09529	*Ammonifex degensii* KC4 YP_003238347	CO dehydrogenase/acetyl-CoA synthase complex; β-subunit	0
kustd1546	CO dehydrogenase/acetyl-CoA synthase; β-subunit	653aa	TIGR01702 COG1151 pfam03063	*Ammonifex degensii* KC4 YP_003238346	CO dehydrogenase, catalytic subunit	2.00E-177
kuste3170	hypothetical phosphotransacetylase protein	356aa	COG0857	*Desulfovibrio desulfuricans* G20 ABB38502.1	cobyrinic acid a,c-diamide synthase family protein	6.00E-67
kuste3344	phenylacetate-CoA ligase (paaK)	435aa	COG1541	*Chitinophaga pinensis* DSM 2588 YP_003121574.1	F390 synthetase-like protein	1.00E-79
kuste4610	CO dehydrogenase/acetyl-CoA synthase; β-subunit	658aa	PF03063	*Thermincola* sp. JR YP_003640687	carbon-monoxide dehydrogenase, catalytic subunit	2.00E-144
kusta0048	acetate-CoA ligase (ADP-forming); β-domain (acdB)	242aa	COG1042	*Cand.* 'Korarchaeum cryptofilum OPF8' YP_001736558.1	hypothetical protein Kcr_0115	5.00E-64
kuste3169	acetyl-CoA synthetase (ADP-forming)	753aa	TIGR02717 COG1042	*Chloroflexus aurantiacus* J10fl YP_001637486.1	acetyl CoA synthetase; α-subunit	0
kustc1128	acetyl-CoA synthetase (acsA)	589aa	PRK04319	*Methanocella paludicola* SANAE YP_003355474.1	acetyl-CoA synthetase	0

Chapter 7

General discussion and outlook

In natural aquatic ecosystems, anammox bacteria are generally limited in one or both of their substrates (ammonium and nitrite), which are intermediate products in several biogeochemical processes that are constantly and often rapidly turned over. Therefore anammox bacteria are often dependent on other nitrogen-transforming processes for the supply of their substrates. Physiological interactions with other microorganisms such as sulfide-oxidizing (partial) denitrifiers or heterotrophic denitrifiers using amino acids were investigated in this thesis (**Chapter 3** and **Chapter 5**). Additionally we showed that organic electron donors (i.e. acetate or amino acids) played a role in anammox metabolism and/or in their interactions with other microorganisms (**Chapter 5** and **Chapter 6**). The environmental study described in **Chapter 2** included a molecular survey of the functional marker genes *hzsA* and *aprA* in the hydrocarbon-rich seeps and hydrothermal vent sediments of the Guaymas basin. The results showed that a diverse group of anammox bacteria of the marine genus *Scalindua* sp. coexisted with sulfide-oxidizing microbes. In **Chapter 4** we studied the effect of different substrate limitations on a coculture of anammox and autotrophic denitrifiers at the gene transcription level. Although the data presented in this thesis give clear insights into the complexity of the nitrogen transformation network anammox bacteria are a part of, more research is needed to understand the dynamics of such interactions and to identify key players in different ecosystems.

Dynamic interactions in a dynamic environment

Although anammox bacteria have been initially enriched from a denitrifying fluidized-bed reactor in which sulfide addition stimulated anammox activity (van de Graaf et al., 1996), later studies often claimed that anammox bacteria were inhibited by sulfide (Dapena-Mora et al., 2007; Jensen et al., 2008; Jin et al., 2013) and therefore absent from sulfidic waters (Dalsgaard et al., 2003; Lam et al., 2007). Recent studies could show however that certain *Scalindua* sp. phylotypes were present in the lower suboxic zone of the Black Sea where sulfide concentrations were up to 10 μM (Wakeham et al. 2012, Fuchsmann et al. 2012, Kirkpatrick et al., 2012). Additionally, Hannig et al. (2007) found that although sulfide-based denitrification was the major N-recycling pathway in the presence of measurable sulfide in the Gotland Deep, anammox activity could account for the majority of N_2 production after an inflow event of oxygenated North Sea water that resulted in a sulfide free zone. This not only implies a certain divergence in the tolerance towards sulfide among different anammox species and that anammox bacteria might be much more versatile and robust than previously assumed, but it also reflects how strongly changes

in the physicochemical conditions can affect the dynamics of denitrification and anammox as potential N_2-releasing processes.
Besides measurable sulfide, a high carbon to nitrogen load has also been reported as a competitive advantage for (heterotrophic) denitrifiers in studies focusing on anammox performance in wastewater treatment plants (Güven et al., 2005, Molinuevo et al., 2009). Accordingly, also anammox activity in natural aquatic ecosystems seemed to be inversely correlated with elevated carbon concentrations derived from organic matter (Engström et al., 2005, Jensen et al., 2005). An exception was found in the Thames river estuary where an increasing sediment organic content positively correlated with the relative contribution of anammox to the release of fixed nitrogen (Trimmer et al., 2003). Although these reports may seem conflicting at first glance, they might be another example of the divergence of the anammox bacteria and rather show niche differentiation of different anammox species, a hypothesis that needs further investigation: Are there different types of anammox bacteria that respond differently to organic carbon, sulfide, temperature or have different competitive fitness for example with respect to denitrifiers? What is the role of temporal and spatial dynamics of ecosystems in anammox and denitrification cooperation or competition and which factors favor which process? And what about the fact that anammox bacteria might very well be able to do much more than combining ammonium and nitrite to dinitrogen gas? What is their metabolic potential? And how would that impact the N-cycle and other biogeochemical cycles? These are few questions that arose during the course of research that resulted in this thesis, some of them have been answered, others are work in progress or should be tackled in near future. Hereafter we will discuss some ideas on anammox ecophysiology and what, how and why it should be investigated further.

Studying the environment with reactor model systems

Anammox bacteria were discovered in a denitrifying fluidized bed reactor, where anaerobic ammonium removal was dependent on nitrate with sulfide and/or organic compounds being the major electron donors in the system (Mulder et al., 1995). Sulfide addition even caused a transient accumulation of nitrite thereby stimulating anammox activity (van de Graaf et al., 1996). This was a first indication that denitrification was supplying nitrite and possibly ammonium for the anammox process. In the environment anammox and denitrification often take place simultaneously (Dalsgaard et al., 2003; Rysgaard et al., 2004; Song et al., 2013), however solid proof that these two processes are actually linked under natural conditions is absent so

far. To study the anammox-denitrifier cooperation in natural ecosystems is difficult as it might be restricted to a very thin layer or specific zones and would require sophisticated and expensive techniques such as NanoSIMS to visualize the exchange of labelled $^{15}NO_2^-$ or $^{15}NH_4^+$. And possibly this could turn out to be an endeavor similar to searching the needle in a haystack. We have chosen for the more straightforward approach of mimicking microbial interactions in reactor systems. This allowed us to follow physiological and molecular responses of our anammox enrichment cultures together with denitrifiers under selected conditions over a long period of time. From this we could show that a stable cooperation between anammox bacteria and (autotrophic/heterotrophic) denitrifiers is indeed possible (**Chapter 3** and **5**). After showing that these interactions are feasible, the dynamics of such an interaction can now be investigated in more detail. Different types of organic nitrogen/reduced sulfur at different or even fluctuating concentrations, ferrous iron as electron donor, influence of oxygen or nutrient limitations might be interesting parameters to test in future experiments. A thorough determination of anammox and denitrifier competition and interaction might have added value for modelling the recycling of fixed N on a larger scale. Ultimately the quest for the 'needle' has to be started by finding suitable ecosystems and showing that such an interaction also plays a role in nature and consequently its environmental relevance should be assessed. In the environment this interaction might be very dynamic depending on a many different variables that can change quickly in time and space and on a very small scale. These variables might include nutrient availability and turnover, organic loading, oxygen or sulfide concentration, mixing or diffusion speed, community composition and activity etc. Our current knowledge on anammox and (heterotrophic) denitrifier physiology would suggest that a system with fluctuating remineralization rates that ensures 'feast and famine' might support activity of anammox bacteria - given sulfide concentrations stay in the low μM range. When concentrations of remineralization products would be constantly high there would be competition for electron acceptor. Periodically high concentrations would favor the oxidation of organic substrates by denitrification and DNRA releasing ammonium. Whereas anammox activity would be favored in times of organic electron donor limitation. Sediment interfaces, water bodies with regular inflow or mixing events, marine upwelling zones, or lakes with cyclic primary production and incomplete mixing might be environments were such a stable interaction could occur. However, an environment where the concentration of electron donors such as organic C, reduced Fe or sulfide exceeds the availability of nitrite

or nitrate would probably support complete denitrification and no or little anammox activity due to electron acceptor competition and/or inhibition. In short, from our current knowledge we hypothesize that excess nitrate and a high sulfide flux in the presence of ammonium would be beneficial conditions for an anammox/autotrophic denitrifier interaction and a low or episodically elevated organic C/N load combined with excess nitrate could be favorable for an anammox/heterotrophic denitrifier cooperation.

Versatile anammox metabolism

Previous studies have shown that anammox bacteria are able to perform dissimilatory reduction of nitrate via nitrite to ammonium at 10% of the normal anammox rate with formate as the electron donor (Kartal et al., 2007a). *Brocadia fulgida* and *Anammoxoglobus propionicus* were enriched in the presence of acetate and propionate, respectively and could oxidize these organic acids at higher rates than other anammox microorganisms, out-competing other anammox species and even heterotrophic denitrifiers for nitrite (Kartal et al., 2007b; Kartal et al., 2008). In theory this might not only increase the growth rate and/or yield, but also shows the physiological versatility of anammox bacteria, which could increase the chances of survival in a dynamic environment. Organic acid oxidation coupled to dissimilatory nitrate reduction to ammonium (DNRA) is an intrinsic property of all anammox bacteria studied so far (Kartal et al., 2007b, 2008, van de Vossenberg et al., 2013). In **Chapter 5** we investigated whether amino acids could serve as ammonium source as they represent an important degradation product of organic matter. Anammox might be able to use amino acids as electron donor for the reduction nitrate/nitrite to ammonium or as ammonium source by deamination of the amino acids. Our data clearly showed that *Kuenenia stuttgartiensis* used ammonium liberated by amino acids deamination in a co-culture with denitrifiers, but could not provide conclusive results on the direct use of amino acids of this anammox species. Whether they are able to use amino acids as electron donor, carbon- and/or ammonium source themselves or rely on other bacteria like fermenters or denitrifiers is not yet clear. To solve this question experiments should be repeated feeding only very low amounts of amino acids to an ammonium-limited reactor system. As we saw immediate consumption of amino acids and $^{29}N_2$ production from ^{15}N-amino acids in our experiments it is plausible to assume that anammox bacteria are able to use amino acids. Feeding low concentrations would keep the actual amino acid concentration in the reactor 0 and thereby constrain the growth of heterotrophic community members provided that anammox bacteria have

a higher affinity to the amino acids than denitrifiers. This would allow much more precise stoichiometric analyses. Several additional experiments could be performed to elucidate the potential of *K. stuttgartiensis* to metabolize amino acids. Looking at the differential expression of genes could help to identify candidate genes that might be involved in amino acid metabolism by comparing the transcriptome of standard-grown, ammonium-limited and amino acid-fed cells. Another interesting puzzle that remains is the fate of the carbon atoms in the amino acids supplied. Experiments conducted previously pointed to CO_2 as the main product of organic acid oxidation (Güven et al., 2005; Kartal et al., 2007b). Despite the fact that formate, acetate and also amino acids could serve as a supplementary carbon source by delivering valuable intermediates in the acetyl-CoA pathway, anammox bacteria seem to continue to fix CO_2 according to natural isotope fractionation (Kartal et al., 2008). However, these experiments should be repeated labelling a whole reactor system over at least one or two division cycles (1-2 weeks). Supplying anammox reactors with ^{13}C-labelled organic acids could show whether carbon groups derived from these compounds are incorporated directly into biomass by analyzing the ^{13}C enrichment in ladderane lipids. The presence of a high $^{12}CO_2$ background and an additional control experiment with a similar culture feeding $^{13}CO_2$ should be able to exclude false positive results in case ^{13}C-labelled organic acids being first converted to $^{13}CO_2$ and then fixed and incorporated. A very similar experimental setup could also be used to answer the question whether amino acids are taken up by anammox bacteria or by potential interaction partners. Using a high $^{12}CO_2$ background ^{13}C-labelled and/or ^{14}C-labelled amino acids could be supplied and organisms taking up the radioactively labelled compound (^{14}C) could be identified by MAR-FISH (McIlroy et al., 2010) or NanoSIMS (^{13}C). Simultaneously feeding ^{15}N-labelled amino acids makes correlation of the ^{13}C and ^{15}N label possible, showing whether the whole amino acids are taken up or whether amino acids are first cleaved and then the N- or C-group is taken up preferentially. The high $^{12}CO_2$ background and time series sampling should minimize the effect of cross contamination of $^{13}CO_2$ re-fixation by anammox bacteria. Similar experiments could also be performed with anammox cells grown on acetate and other organic acids. Although previous studies could show that organic acids can be used in the nitrate reduction pathway to ammonium (Güven et al., 2005; Kartal et al., 2007b) and also our own experiments presented in **Chapter 6** could confirm that the highest expressed AMP-forming acetyl-CoA synthetase gene (*acs*) from *K. stuttgartiensis* can activate acetate to acetyl-CoA it needs to be shown whether anammox bacteria can also grow

mixotrophically.
With regard to the versatile metabolism and the potential adaptation of different anammox bacteria to their environment also the potential of other anammox species to grow on amino acids should be tested: The *Scalindua* genus would be interesting as *Sc. profunda* and *Sc. brodae* genomes have been sequenced and both encode a number of oligopeptide transport systems that were absent from the *K. stuttgartiensis* genome (van de Vossenberg et al., 2012; Speth et al., 2015) and might point to them using organic nitrogen.
All in all, we have to confess that some of these questions can only be answered with pure culture studies.

Anammox niche differentiation

What other physiological properties could make a particular anammox species more suitable to survive in certain environments? These might include oxygen tolerance, sulfide tolerance, organic acid oxidation, adaptations to high or low temperatures or substrate concentrations. Answering these questions will be challenging as physiological experiments would need to be performed with many different anammox bacteria. Enriching biomass from environmentally relevant species for comparative physiology studies might be difficult due to sampling limitations (water column biomass is more difficult to acquire) and the fact that anammox bacteria are generally tedious to cultivate. However, large scale genome sequencing and maybe even single cell sequencing of relevant ecosystems might open up the possibility of gaining a first glimpse into anammox niche differentiation by comparing their genomes. In those few cultivated species of which the genome has been analyzed we already encounter many genomic features that raise questions. An example is the nitrite reduction pathway in different anammox bacteria: *Scalindua profunda* encodes for a cytochrome cd_1 type enzyme (NirS) to reduce nitrite to NO and it is also one of the highly expressed proteins (van de Vossenberg et al., 2013). *Kuenenia stuttgartiensis* also encodes this enzyme, but expression levels are very low on both transcriptomic as well as protein level (Kartal 2011). This led to the hypothesis that another enzyme might also be capable of reducing nitrite to NO, possibly a hydroxylamine oxidoreductase (HAO)-like protein encoded in all anammox bacteria studied so far (Kartal et al., 2013). Furthermore, the anammox bacterium *Jettenia caeni* and the very closely related *Jettenia asiatica* contain the copper-containing nitrite reductase gene (*nirK*) (Hira et al., 2012; Hu et al., 2012) and in *Brocadia fulgida* and *Brocadia sinica* the conventional nitrite reductase seems to be absent (Gori et al., 2011, M. Oshiki personal communication). It would be interesting to know why

certain anammox genera employ different enzymes for nitrite reduction, not only from an evolutionary point of view, but also with regard to their physiology and trace element requirements. Do different nitrite reduction pathways also help explaining anammox niche differentiation by having different affinities for example? For *Brocadia sinica* and *Brocadia anammoxidans* lacking the conventional *nirS* or *nirK* gene the affinity for nitrite differs considerably with <5 μM for *Brocadia anammoxidans* (Strous et al., 1999a) and 86±4 μM for *Brocadia sinica* (Oshiki et al., 2011). That is why especially the HAO-like protein should be purified and its role in anammox metabolism determined. Is it able to reduce nitrite to NO and at what rate? And to what extent does it contribute to nitrite reduction if *nirS* is also expressed, like in *Scalindua profunda* under standard growth conditions (van de Vossenberg et al., 2008)? Unfortunately it is not possible to make a knockout mutant of these genes to test their redundancy in *Scalindua* sp. as there is no genetic system is available for anammox bacteria, yet.

The restricted phylogenetic diversity in marine ecosystems remains a very interesting question as the determining factor selecting for marine *Scalindua* and freshwater species has not yet been found and would represent another important characteristic defining an anammox niche. In **Chapter 2** we exclusively found *hzsA* amplicons related to the *Scalindua* genus in the Guaymas Basin sediment. Also other studies identified almost exclusively *Scalindua* sp. molecular markers in marine ecosystems (Schmid et al., 2007; Woebken et al., 2008). What are the factors that determine the predominance of *Scalindua* sp. in marine ecosystems and what defines growth of *Kuenenia*, *Brocadia* or *Jettenia*? The answer to this seems to be more complex than just the presence of salt, as freshwater anammox species adapt quickly to elevated salt concentrations (Kartal el al., 2006). A combination of a marine (*S. profunda*) and a freshwater (*K. stuttgartiensis*) anammox species in a membrane bioreactor under competitive conditions (Yan, 2012) was used previously to find the determining factors. This study could identify low temperature and nitrite limitation as favorable conditions for the marine anammox bacteria whereas *K. stuttgartiensis* appeared to be the better competitor for ammonium. In the scope of this thesis we also, contributed to this discussion with our results from **Chapters 3 & 4**. The reactor system described in **Chapter 3** was inoculated with anammox biomass that consisted of *K. stuttgartiensis* and *S. profunda* in equal amounts. After several months of operation the metagenome taken from this reactor showed a clear dominance of *K. stuttgartiensis*. After 1.5 years of operation when samples were taken for comparative transcriptomics (**Chapter 4**) it was found that *K. stuttgartienis* could not be detected anymore

(FISH and matched reads <0.04%) and *Scalindua profunda* was the dominant anammox bacterium. One possible explanation could be the low ammonium concentrations (40 μM) in the reactor before and during the first experiment, but that does not agree with previous results (Yan, 2012). Additionally fluctuating room temperature conditions, nitrite limitation, prolonged exposure to elevated salt concentrations (2.5%), successful flock formation, the presence of an active sulfur cycle etc. might have also contributed to the population shift. An attempt to answer this question could be a parallel startup of several reactor systems inoculated with the same starting material (planktonic cells) of freshwater and marine anammox species in a 1:1 ratio. Each reactor system would be changed in only a single parameter, such as nitrite, ammonium, temperature, organic nitrogen or salt concentration etc. to get an idea of the factors that contribute to freshwater/marine anammox dominance over a prolonged period of time. Answering this question is very difficult as it is probably based on the combination of facts discussed in this outlook i.e. dynamic interactions with other microorganisms, genetic adaptation and resulting niche differentiation and metabolic diversity.

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–from Star Trek TNG

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Curriculum vitae

Lina Russ was born January 29th 1986 in Goch, Germany. After completion of her secondary education at the Konrad-Adenauer-Gymnasium Kleve in 2005, she studied biology at the Radboud University in Nijmegen (NL). During her studies she specialized in microbiology and did two internships (BSc and MSc) at the RU Department of Ecological Microbiology. In her first internship she applied qPCR to detect and quantify the NC10-like *pmoA* gene in an enrichment culture and the second intership involved molecular and biochemical techniques to investigate the *Acidianus* sp. CS_2 hydrolase, leading to a high-impact publication. The final year of her master she spend at the ETH in Zurich, Switzerland to work on two projects in the group of Prof.dr. Julia Vorholt: The optimization of the high-throughput identification of methanotrophic isolates in the rice phyllosphere and making use of comparative genomics to identify possible determinants for a plant-associated lifestyle in eight *Methylobacterium* sp. genomes. After her graduation in august 2010 she started working as a PhD student in the group of Prof.dr.ir. Mike Jetten. Under the supervision of Dr. Boran Kartal and Dr. Huub op den Camp she studied the physiology and ecology of anaerobic ammonium oxidizing bacteria which resulted in the present thesis. Since april 2015 she is working on biological control products at Plant Research International in Wageningen.

Publications

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